Isolation and characterisation of Lolium perenne genes involved in fructan metabolism

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Publication date:
2007

Document version
Publisher's PDF, also known as Version of record

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Ph.D thesis by

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Store Heddinge 2007

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Front cover
High-fructan transgenic line (right) and
control line (left) in F6 genotype

Preface

The present PhD thesis was initiated through the biotechnology consortium between DLF-TRIFOLIUM and Risø National Laboratory and was performed through the Royal Veterinary- and Agricultural University, Copenhagen. The PhD programme was funded by the DLF-Risø biotechnology consortium.

The fructan tropic was a new subject in the DLF-Risø consortium and was initiated with this PhD project. However, before I started, PhD Morten Storgaard was performing the constructs with heterologous fructosyltransferases for transformation in *L. perenne* and for this and subsequent discussion, I wish to address a special thank to him. In addition, a special thank goes to the transformation group at Danish Plant Breeding station (DP) lead by PhD Marianne Folling and to PhD Klaus Petersen for introduction of Q-PCR and for critical discussion through this thesis.

I thank my supervisors Professor David B. Collinge and PhD Thomas Didion and also head of research Klaus K. Nielsen for their guidance through this thesis. I am very grateful to PhD Thomas Didion for critical reading of this thesis and inspiring guidance and discussions throughout this thesis. Additionally, I wish to thank all former and present scientists and technicians at Risø and DP for their help and general encouragement.

Finally, I would especially like to thank Jakob Arvad for his personal support and encouragement during the entire study.

Gitte Gadegaard Larsen
April 2007

Summary

Fructans are the main storage carbohydrate in grasses and are metabolised by catalysis of a number of fructosyltransferases (FTs) and fructan exohydrolases (FEHs). In *L. perenne*, three different fructan classes have been identified based on their structures. So far, the cloning of genes encoding FTs from *L. perenne* account only for the synthesis of two of these classes. The FT(s) responsible for synthesis of the last fructan class thus remains to be identified. Furthermore, no genes encoding FEHs from *L. perenne* have been functionally characterised and the role of FEHs in the regulation of fructans is currently unknown.

Using transgenic approaches, bacterial and plant FTs have been transformed into both non-fructan- and fructan-accumulating plants and increased fructan content has been demonstrated. One of the objectives in the present PhD thesis was to investigate the possibility of increasing the fructan content in transgene *L. perenne* lines. The second objective was to investigate the seasonal variation of the water-soluble carbohydrates (WSC) and fructan content during a field experiment. The third objective was to clone and perform molecular characterisation of FTs and FEHs.

For the first objective, experiments were performed and showed up to 3-fold improvement of the fructan content in transgenic *L. perenne*. Furthermore, it was demonstrated that the fructan content, besides for being increased, also was more stable throughout the investigated growth period; a feature which was not detected in traditionally bred variety with high sugar content. Although no correlation between the fructan content and transcription levels of neither the introduced/homologous FTs nor the transgene copy number could be detected, the investigations clearly showed that transgene transcription was a prerequisite for obtaining high fructan levels.

Seven *L. perenne* varieties were analysed for WSC and fructan content through the growth season in 2004 in two independent establishment years and repeated again in 2005 to fulfil the second objective. Independently of the sward age of the plots, all the varieties contained high WSC and fructan content during the summer, which decreased through the autumn depending on the establishment year. However, the WSC and fructan content was different in the two growth seasons and especially, the accumulation of fructans was higher in 2005 than 2004. This is probably a result of the low precipitation in 2005, since fructans are proposed to be accumulated under drought conditions. Based on the investigation, recommendations of selected elite varieties were performed resulting in production of a new grass mixture used to grazing of horses.

In order to fulfil the third objective, cloning of genes involved in fructan metabolism were performed and resulted in isolation of two and six putative FTs and FEHs, respectively. One of the FTs (*LpFTa*) exhibited high sequence identity to another *L. perenne* gene, where the gene has been demonstrated to display 1-SST activity. The other isolated FT (*LpFTb*) did also exhibit high sequence identity to other *L. perenne* genes but no functional characterisation for the gene encoding the FT has been performed yet. It is however possible that *LpFTb* is involved in the synthesis of the fructan class, where the enzyme responsible for the synthesis in *L. perenne* is unknown.

The six putative FEH genes showed sequence identifies to an uncharacterised FEH from *L. perenne.* Two out of the six isolated genes showed an unknown feature as they are assumed to be a spliced variant of a full length FEH, where the 5’ and 3’ end shows 96 to 100 % sequence identities to a full length clone.

Sammendrag

Fruktaner udgør hovedparten af kulhydratlageret i græsser og er metaboliseret under katalyse af et antal forskellige fruktosyltransferaser (FT) og fruktan exohydrolaser (FEH). Tre forskellige fruktan klasser er blevet identificeret i *L. perenne* baseret på deres struktur. Kloningen af generne, der koder for FT fra *L. perenne*, omfatter indtil videre kun de FT enzymer, der syntiserer to af klasserne. De/den FT, der er involveret i syntesen af den sidste fruktan klasse, mangler således stadigvæk at blive identificeret. Derudover findes der ingen funktionel karakterisering af de gener, der koder for FEH i *L. perenne*, og rollen for FEH i reguleringen af fruktaner er indtil videre ukendt.

Ved at anvende transgene fremgangsmåder er FT fra bakterier og planter blevet transformeret ind i både ikke-fruktan og fruktan-akkumulerende planter og forøget fruktan indhold er blevet demonstreret. En af målsætningerne med denne ph.d. afhandling var at undersøge, om det er muligt at forøge fruktan indholdet i transgene *L. perenne* planter. Den anden målsætning var at undersøge det sæsonvarierende indhold af vandopløselige kulhydrater (WSC) og fruktan indholdet i mark forsøg. Den tredje målsætning var at klone og udføre molekylære karakterisering på de gener, der koder for FT'er og FEH'er.

For at undersøge den første målsætning, blev eksperimenter gennemført, der viste op til 3-gange forøgelse af fruktan indholdet i transgene *L. perenne*. Derudover blev det demonstreret, at fruktan indholdet, udover at være forøget, også var mere stabilt gennem den undersøgte vækst periode; et kendetegn, som ikke kunne måles i en traditionel forædlet sort med højt sukker indhold. På trods af, at der hverken kunne findes en sammenhæng mellem fruktan indholdet og transskriptions niveauet af de introducerede eller de homologe FT-gener og eller det transgene kopi antal, viste eksperimenterne tydeligt, at transgen transskription var nødvendig for at opnå de høje fruktan niveauer.

For at undersøge den anden målsætning blev syv *L. perenne* sorter analyseret for indholdet af WSC og fruktan gennem vækstsæsonen i 2004 med to uafhængige udlægs år. Dette eksperiment blev gentaget igen i 2005. Uafhængigt af parcellernes alder havde alle sorterne et højt WSC- og fruktan indhold om sommeren. Fruktan indholdet faldt i efteråret for alle sorterne, men forskellene i fruktan indholdet var afhængigt af udlægs året. Der var forskel i WSC og fruktan indholdet i de to vækst sæsoner og især akkumuleringen af fruktan var højere i 2005 sammenlignet med 2004. Dette skyldes formentlig den lave mængde af nedbør i 2005 idet fruktaner forventes at blive akkumuleret som følge af tørke. Ud fra disse undersøgelser blev udvalgte sorter anbefalet og en ny græs blanding til hestehold blev introduceret.

Til undersøgelse af den tredje målsætning blev gener, der er involveret i fruktan metabolismen, klonet og to formodede FT'er og seks formodede FEH'er blev molekylært karakteriseret. En af de isolerede FT'er (*LpFTa*) havde høj sekvens identitet til et anden *L. perenne* gen, som er vist at have 1-SST aktivitet. Den anden isolerede FT (*LpFTb*) havde også høj sekvens identitet til andre formodede *L. perenne* FT gener, men disse er endnu ikke blevet funktionel karakteriseret. Det er derfor muligt, at *LpFTb* er involveret i syntesen af den fruktan klasse, hvor det enzym, der er ansvarlig for syntesen i *L. perenne*, endnu ikke er kendt.

De seks formodede FEH gener viste sekvens identitet til en ukarakteriseret FEH fra *L. perenne*. To ud af de seks isolerede gener viste et ukendt kendetegn, idet de formodes at være splejset udgaver af en fuldlængde FEH, hvor 5' og 3' enden viste mellem 96 til 100 % sekvens identitet til fuldlængde klonen.

Content

Abbreviation list

1-FFT:	fructan:fructan 1-fructosyltransferase
1-SST:	sucrose:sucrose 1-fructosyltransferase
6-FT:	fructan 6-fructosyltransferase
6G-FFT	fructan:fructan 6G-frucosyltransferase
6-KES:	6-kestosidase
6-SFT:	sucrose:fructan 6-fructosyltransferase
AI:	acid invertase
A/NI:	alkaline/neutral invertase
DP:	degree of polymerisation
FEHs:	fructan exohydrolases
FRU:	fructose
FTs:	fructosyltransferases
GLC:	glucose
INV:	Invertase
L.perenne:	*Lolium perenne*
SacB:	gene encoding levansucrase from *Bacillus subtilis*
TLC:	Thin Layer Chromatography
WSC:	water-soluble carbohydrate

1. Introduction

Perennial ryegrass (Lolium perenne L.) is one of the most important forage crops in the temperate regions. Although highly valued for its high palatability and digestibility, the nutritional value of grass is lower than other high quality fodders such as maize silage. Grasses contain an insufficient amount of carbohydrates for optimal utilisation of the micro-organisms in the rumen of the ruminants. As a consequence, suboptimal milk and meat production occurs with an excretion of ammonia to the environment. Increasing the amount of carbohydrates in grasses does not only decrease the release of ammonia but results also in improved milk and meat production.

Fructan is the main storage carbohydrate in grasses and is primary stored in stems, though it is also present in the leaves and roots. Fructans are synthesised from sucrose by the action of several fructosyltransferases (FTs) and can be degraded by different fructan exohydrolases (FEHs). In general, five different classes of fructans have been described in plants but only three classes are identified in *L. perenne*. At the time this PhD project was initiated, only two FT genes and no FEH gene have been cloned from *L. perenne*.

The present PhD thesis comprises the results of three years investigations in order to investigate the following objectives:

I) Improvement of the fructan content in transgenic *L. perenne* by over-expression of heterologous FT genes
II) Seasonal changes of the water-soluble carbohydrates (WSC) content in DLF-TRIFOLIUMs elite varieties
III) Cloning and molecular analysis of genes encoding FTs or FEHs isolated from *L. perenne*

This PhD thesis consists of a review of the literature in the field of fructan research and an experimental part. The review part (chapter 2) provides an overview of the current knowledge of the FTs and FEHs involved in fructan metabolism in plants and especially *L. perenne*. This is followed by a description and discussion of published studies using transgenic approaches to increase fructan content.

The experimental part (chapter 3 and 4) contains three papers and additional data supporting one of the papers. The first submitted paper describes the development of transgenic grass lines which exhibit up to 3-fold higher fructan content. The second paper draft describes the WSC and the fructan content in seven elite varieties of *L. perenne* grown under two different growth seasons and with different sward age. The third paper is a Danish paper published in Tölt, a magazine for Icelandic ponies, which describes the fructan content in a low and high-fructan varieties and its possible correlation to the disease, laminitis.

Finally, conclusions are made based on the data presented and perspectives for further work are discussed in chapter 5.

2. Fructan metabolism

Carbohydrates are central to the carbon and energy needed by the plants and sucrose is of particular importance due to its role as major currency of energy exchange between source and sink tissue in higher plants (Foyer *et al.*, 2000; Pollock *et al.*, 1995).

The functions of carbohydrates in plants are not only to act as substrates for growth but also to be messengers affecting carbohydrate-sensing systems that initiate changes in gene expression (Foyer *et al.*, 2000; Koch, 1996). Some carbohydrates can enhance or repress expression of genes, while other carbohydrates are ineffective. Recently, a study using Arabidopsis leaves, incubated with sucrose, showed that 337 genes were induced and 307 were repressed, illustrating the huge importance of carbohydrate for gene regulation in the plants (Muller *et al.*, 2007). The response will vary depending on the specific carbohydrate even though metabolic flux may be more important than the actual level of carbon resources. The responses are unique in plants because changes in carbohydrate allocation can ultimately modulate form through processes affecting import/export balance. The carbohydrate-regulated genes in plants represent a mechanism to adjust metabolism according to environmental changes. In leaves of *Lolium* changes in gene expression related to storage and carbon use are observed when carbohydrate levels rise (Winters *et al*., *1994*).

Induction of FT expression has been investigated in barley and wheat and in both species the expression was induced by sucrose and trehalose (Noel *et al.*, 2001; Muller *et al.*, 2000). Glucose and fructose lead to a weaker induction indicating a disaccharide-mediated regulation of FTs in the two species. Sucrose sensing in the same two species has been demonstrated to be hexokinase independent and both protein kinase and protein phosphatase activities are involved in the pathways that affect the induction of fructan synthesis by sugars (Noel *et al.*, 2001). Also nitrate has been reported to be a negative signal for fructan metabolism and this regulation network is independent from the sugar signalling pathway (Morcuende *et al.*, 2004).

Formation of sucrose involves several steps; first, glyceraldehydes 3-phospate (also called Triose-P) is synthesised as a result of the *Calvin cycle* where CO_2 is converted into Triose-P during the photosynthesis in the chloroplast cells (figure 2.1). Triose-P can be converted to starch in the chloroplast but most of the Triose-P is transported to the cytoplasm. Secondly, Triose-P is converted to sucrose through a series of reactions.

The regulation of photosynthetic carbon metabolism is extended to differentiate between species regarding whether starch or sucrose is the major storage compound (Pollock, 1986).

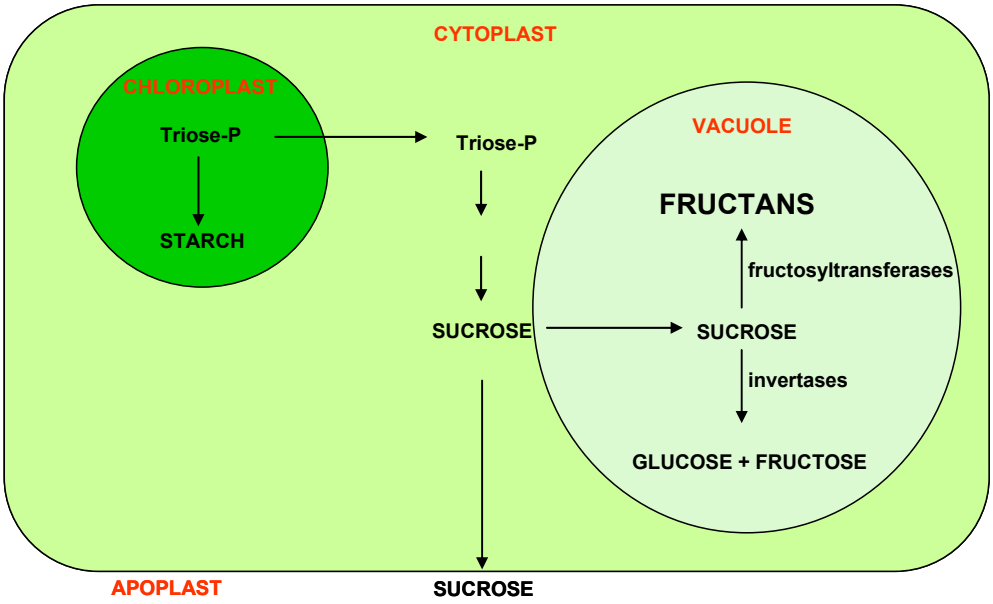

Figure 2.1 Illustration of carbohydrate metabolism in a plant cell.
Sucrose from the cytoplast is either transported to the apoplast or the vacuole. In the vacuole, sucrose is either hydrolysed to glucose and fructose by the catalysis of invertases or used in fructan metabolism. Modified from Vijn and Smeekens (1999).

Starch is the most common leaf carbohydrate reserve in plants but not in grasses, where fructans function as primary carbohydrate storages (Cairns *et al.*, 2002b). However, starch can be found in high concentration in grass seeds (47 % of DW), but why starch is a minor component of the carbohydrate reserve in grasses is not known (Cairns, 2002; Cairns *et al.*, 2002a). It has been suggested that the low capacity for starch accumulation is a result of evolution of fructan metabolism as a result of low chloroplast capacity for starch accumulation (Cairns *et al.*, 2002b). Experiments to investigate the role of fructan and sucrose synthesis on starch accumulation has been performed in leaves of *L. temulentum* (Cairns *et al.*, 2002b). Interestingly, blocking the fructan metabolism did not enhance starch synthesis. This suggests the existence of two independent synthesis pathways; one for fructan and one for starch (Cairns, 2002). However, blocking the sucrose synthesis resulted in the inhibition of both fructan and starch metabolism, demonstrating that sucrose is the preliminary substrate for both metabolisms. Furthermore, sucrose accumulation did not result in enhanced starch synthesis.

Normally, starch metabolism is regulated by a sucrose-mediated feedback inhibition via phosphate transport and this sucrose mediated feedback inhibition is apparently not occurring in grass leaves (Cairns *et al*., 2000). The missing regulation of starch metabolism in grasses could be due to the fact that grasses are a fructan accumulating plant. Elements of the regulatory cycle of sucrose synthesis in starch accumulating species are, however, also present in grasses (Pollock *et al.*, 1995). Using leaves of *Lolium temulentum*, Fructose 2,6 bisphoshate (Fru-2,6-P_2) increased in concentration, when the export of sucrose was blocked (Draborg *et al.*, 2001; Pollock *et al.*, 1989; Housley and Pollock, 1985). Fru-2,6-P_2 is a key regulator of the carbohydrate metabolism and regulates the fixed carbon between sucrose and starch (Stitt, 1990).

Fructose-6-P2kinase/fructose-2,6-bisphosphatase (F2KP) is the enzyme which synthesises Fru-2,6-P2 and using transgenic Arabidopsis plants with reduced F2KP activity, it could be demonstrated that Fru-2,6-P_2 affects the photosynthetic carbon and thereby demonstrating that Fru-2,6-P_2 is a regulator of photosynthetic carbon metabolism since reduced levels of Fru-2,6-P_2 forces an increased flux of carbon into sucrose synthesis during photosynthesis (Rung *et al.*, 2004; Draborg *et al.*, 2001). Since sucrose is the sole substrate for fructan metabolism, the use of Fru-2,6-P_2 could be a strategy for increasing the sucrose content and thereby also the possibility of increasing the fructan metabolism.

Fructan chains are synthesised from sucrose through the action of a number of FTs by adding fructose molecules to the growing chain composing five different classes of fructans. Degradation of fructans is initiated at the end of the growing chain and is catalysed by FEHs. Figure 2.2 gives an overview of the different fructans, which are synthesised or degraded in plants.

The following chapter describes the current knowledge generated on fructan metabolism in plants with a special emphasis on perennial ryegrass, *Lolium perenne*. Furthermore, the different classes of fructans identified, as well as the cloning of FTs and FEHs and their enzymatic function, especially in grasses will be discussed.

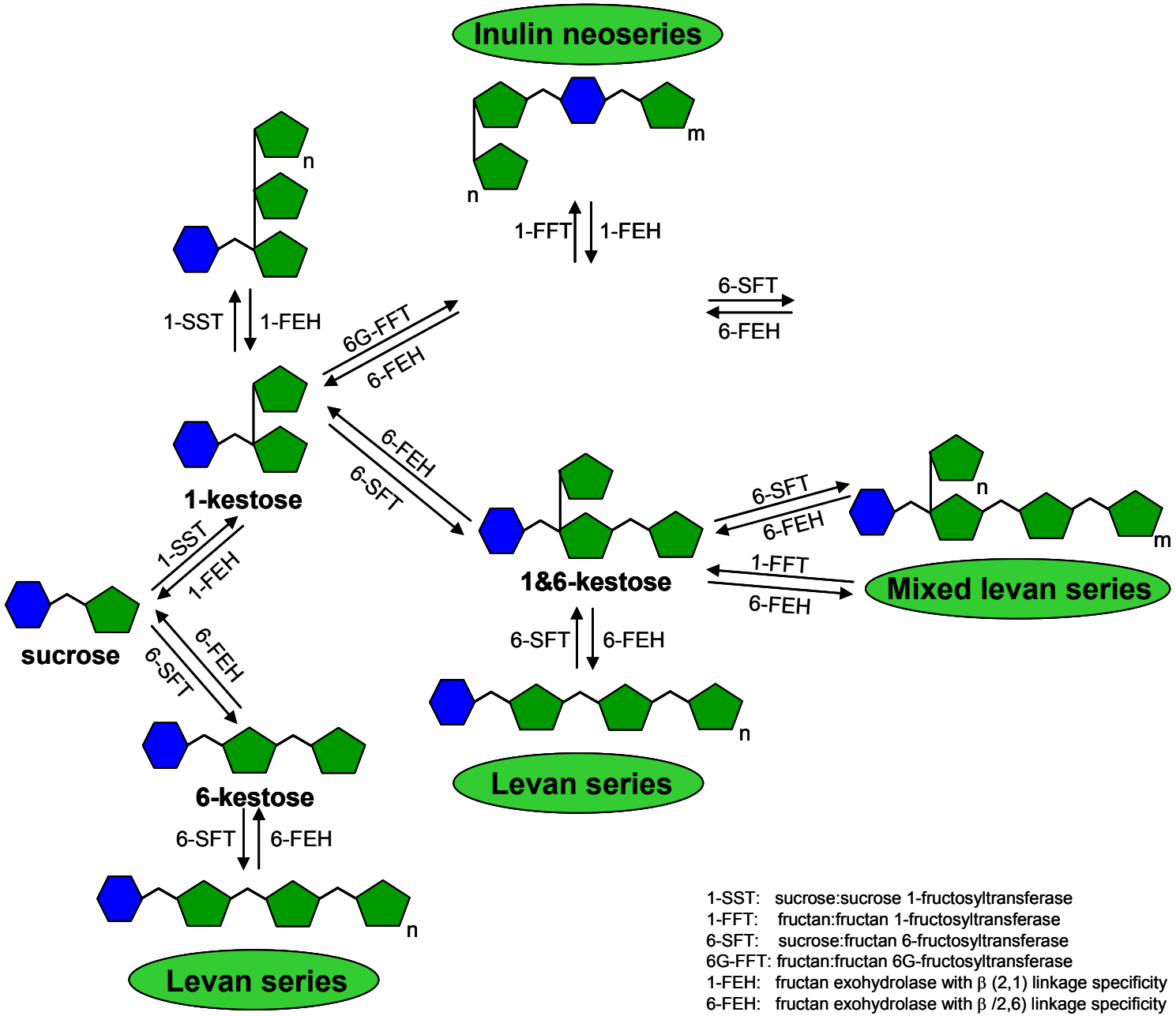

Figure 2.2 Model of the fructan metabolism in plants.
Sucrose is the initiating substrate and by the action of several fructosyl-transferases, five different fructan classes can be synthesised.
Opposite, fructan exohydrolases can degrade the synthesised fructans.
m and n represent different number of fructose units.

2.1 *Lolium perenne* as an important forage species

2.1.1 *Lolium perenne*

The grass family, *Poaceae* is a large and widespread family of plants including economically important species such as wheat, rice, maize, rye, barley, sugarcane as well as the subject of this study, ryegrass. However, not all grasses accumulate fructans as their main carbohydrate storage components. Rice, maize and sugarcane are examples of non-fructan accumulating plant species, whereas wheat, rye, barley and ryegrass all are able to synthesise and accumulate fructans.

Lolium perenne L. is a cool-season perennial ryegrass and is characterised by having high digestibility and palatability and together with fast establishment and high yield potential (Delagarde *et al.*, 2000; Hannaway *et al.*, 1999). These characteristics make the species highly valued for dairy and sheep forage systems and it is therefore primarily grown for pasture and silage (Hannaway *et al.*, 1999; Hubbard, 1992).

The optimal growth temperature of *L. perenne* L. is 20-25 °C (Hannaway *et al.*, 1999). At higher temperatures, perennial ryegrass becomes dormant. Perennial ryegrass is more sensitive to changes in temperatures and drought than annual ryegrass, e.g. *Lolium multiflorum*. Grazing or cutting influences not only the persistence but also the quality and the productivity. Nevertheless, perennial ryegrass can withstand frequent grazing and is thus ideal for intensive grazing systems. Seedlings must be 25-30 cm high before first harvest or grazing. Grasses must be allowed to re-grow to a size of 10-25 cm before next grazing or harvesting. Mechanical harvest is optimal with a cutting height of 5 cm above the ground (Fulkerson and Donaghy, 2001; Hannaway *et al.*, 1999). Perennial ryegrass accumulates high levels of carbohydrates in spring and autumn but repeated harvesting without allowing replenishment of stored carbohydrates reduces the persistence and the re-grow abilities (Hannaway *et al.*, 1999). Accumulation of fructans is favoured by conditions, which stimulate carbon fixation, e.g. long photoperiod. Thereby, the fructan content is higher at four p.m. compared to the levels at 9 a.m. Opposite, the fructan accumulation is minimized when the growth is stimulated, e.g. frequent defoliation.

A *L. perenne* tiller is composed of a leaf and a stem with the new leaf elongates from inside of the tiller (figure 2.3).

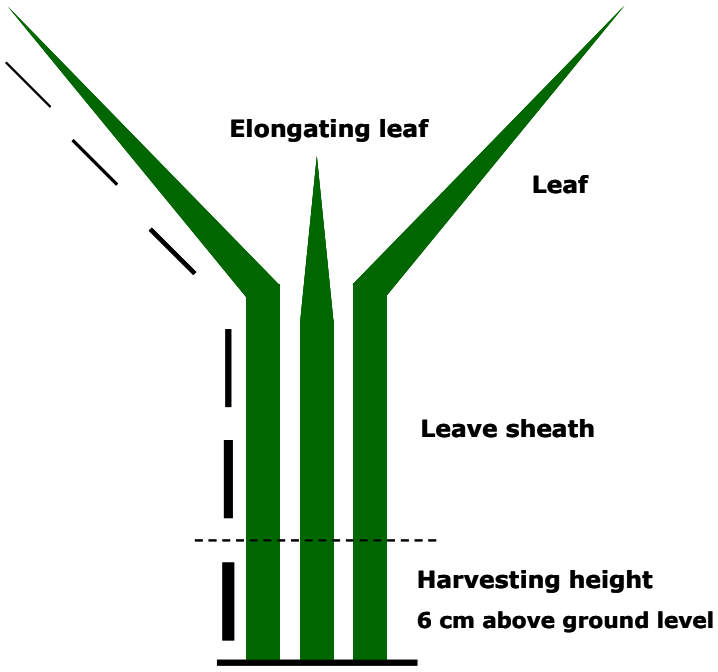

Figure 2.3 Schematic view of *Lolium perenne*.
The fructan content is lowest in the tip of the leaf and is increasing through the leaf and stem illustrated by increasing thickness of the squares. Harvest height is illustrated by a dotted line. Modified from Pavis *et al*. (2001a).

The growing part of the leaf is enclosed by its basal region, which is surrounded by the sheaths of older leaves. The major storage organ in grasses is the stems, where up to 70 % of total fructan content of the vegetative part is stored (Morvan-Bertrand *et al.*, 2001). It has been demonstrated that the fructan content is lowest in the tip of the leaf and increases through the leaf to the stem and is highest in the bottom of the stem illustrated by arrows in figure 2.3 (Pavis *et al.*, 2001a).

Transformation of monocots like *L. perenne* is, in general, performed by micro projectile bombardment of callus where the DNA is coated to heavy metal particles (Altpeter *et al.*, 2000; Spangenberg *et al.*, 1995). As a result of bombardment, the DNA is transferred through the plant cell wall and membranes into the nucleus, where transgene integration in the plant genome takes place. An advantage of this method is the high generation rate of transgenic lines but the lines can show variation within degree of rearrangement, transgene integration and gene silencing and these features are some of the factors that can contribute to variable transgene expression levels (Svitashev *et al.*, 2000).

L. perenne transformation is very time consuming and has a time frame of almost one year due to a 12 weeks long vernalisation period and additional 12-16 weeks from vernalisation to the transgenic plants begin to produce flowers (Altpeter *et al.*, 2000). For comparison, it only takes 16 weeks for generation of T1 plants after transformation of the model plant Arabidopsis (Somerville and Koornneef, 2002).

2.1.2 Nutritional value of grasses

The feeding value of grass or other plants is a product of the concentration of nutrients contained in the forage (nutritional value) plus the amount of forage that an animal will eat (voluntary intake). The voluntary intake together with the digestibility of the fodder is the most important factors in the feeding value, because of their critical role in the supply of energy to the animal. Perennial ryegrass is highly valued for ruminants due to its high palatability (voluntary intake) and digestibility (Delagarde *et al.*, 2000). However, the nutritional value is rather low and is caused by an inefficient utilisation of the dietary proteins in the grass by micro-organisms in the rumen as a result of inadequate carbohydrate content, which supply the micro-organisms with energy.

Proteins in the grass are degraded in the rumen and the micro-organisms use the products of breakdown to grow and to produce more protein that is digested in the small intestine. Ultimately, the microbial protein produced in the rumen and the end-products of the fermentation, provides the cows with an important proportion of protein, nutrient and energy requirements for the production of milk and meat production (Beha *et al.*, 2002; Broderick *et al.*, 2000).

If the grass lacks readily available energy such as carbohydrates, the rumen micro organisms can not grow or will instead use the amino acids to provide energy. Besides using the amino acids, which could have been used to produce protein, the use of amino acids to provide energy, is a wasteful process which results in nitrogen being released from the grass into the rumen as ammonia and later excreted via the urine (Moorby *et al.*, 2006; Lee *et al.*, 2002; Biggs and Hancock, 2001). Ammonia excretion to the environment is a component of nitrogen pollution and is a subject to EU regulations (Kingston-Smith and Thomas, 2003).

The efficiency of protein use in meat and milk production can be increased by feeding the ruminants with energy-rich foods. Another alternative is to increase the amount of carbohydrates in the grass (figure 2.4).

Increasing the carbohydrate content in grass has been demonstrated to increase the conversion of amino acids to proteins (meat and milk) and also to reduce the excess of ammonia to the environment as illustrated in figure 2.4 (Moorby *et al.*, 2006; Lee *et al.*, 2003; Miller *et al.*, 2001).

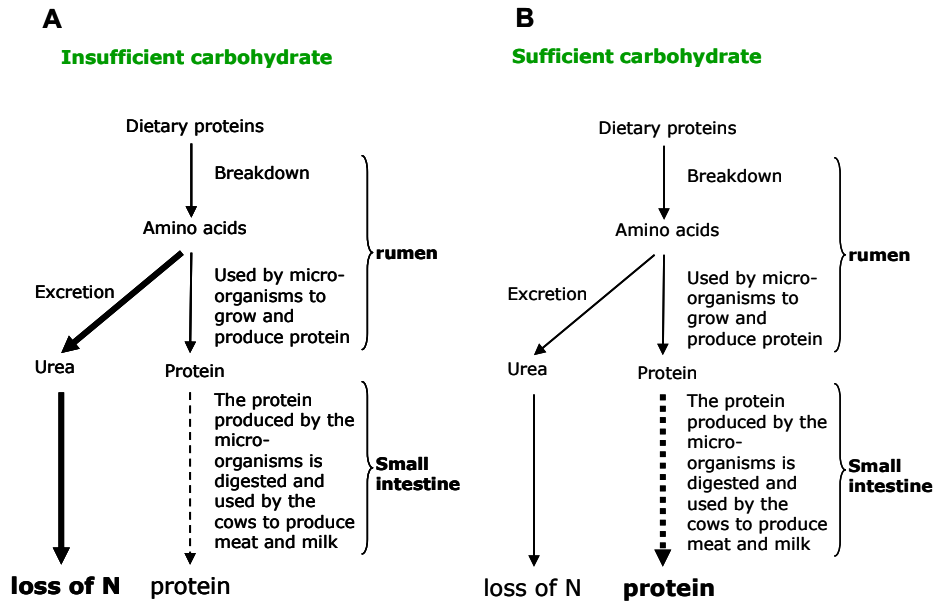

Figure 2.4 The utilisation of carbohydrates in ruminants.
The dietary proteins in the grass fodder are broken down to ammonia in the rumen and used to build up proteins by micro-organisms under use of carbohydrates. If the amount of carbohydrates is insufficient a large excess of urea is excreted and only a small degree of protein is produced in the small intestine (A). If the amount of carbohydrates is sufficient only a small amount of urea is excreted to the environment and an improved amount of protein is produced in the small intestine increasing the production of meat and milk (B).

Also the nitrogen availability in the rumen often exceeds the energy supply which leads to a reduction in the efficiencies of microbial production. This increases the production of rumen ammonia and loss of nitrogen via the urine (Dewhurst *et al.*, 2000). The efficiency of forage nitrogen conversion into meat and milk is only 20-25 % when feeding ruminants with fresh forage, hay and grass silage only (Cairns, 2002).

2.2 Fructan metabolism and the genes involved in fructan metabolism and degradation in *Lolium perenne*

2.2.1 Five fructan classes are identified in plants

Fructans, linear or branched polyfructose molecules, are polymers build up by adding fructose unit to a sucrose unit as a starting point. The added fructose unit is linked at the C2 of one fructose residue and at either the primary alcohol group at C1 or C6 position of a fructose forming β(2,1) or β(2,6) linkages, respectively. The glucose molecule from sucrose can be internal or external depending on the classes of fructans. However, fructan series with no glucose molecules have also been reported from species of the family Asteraceae (*C. intybus* L., *H. tuberosus* L. and *Taraxacum officinale* Weber) although they only represent a minor group (Ernst *et al.*, 1996).

Five different classes of fructans have been identified in plants I) inulin series, II) inulin neoseries, III) levan series, IV) levan neoseries and V) mixed levan, also known as Graminan (Table 2.1).

Table 2.1 The five fructan classes identified in plants.

Class	Linkage type	Example	Present in *L. perenne*[a,b]
Inulin series	β(2,1)	1-kestose	+
Inulin neoseries	β(2,1)	Neo-kestose	+
Levan series	β(2,6)	6-kestose	-
Levan neoseries	β(2,6)	6G-kestose	+
Mixed levan (Graminan)	β(2,1) and β(2,6)	Bifurcose	-

a: Bonnett *et al*. (1994) and b: Sims *et al*. (1992).

Lolium species accumulate mainly fructans with β(2,6) linkages, which are mostly linear but also branched fructans can be observed (Pavis *et al.*, 2001b). In *L. perenne*, fructans from three different classes (inulin series, inulin neoseries and levan neoseries) have been identified (Bonnett *et al.*, 1994; Sims *et al.*, 1992). Similar structures of fructans have been reported from different varieties of *Lolium* (Pavis *et al.*, 2001b). However, the fructan structures can not be used as taxonomic markers for different families. For instance, bifurcose has not been identified in any *Lolium* species but it has been identified in wheat and barley, which are members of the *Poaceae* family (Pavis *et al.*, 2001b; Chatterton *et al.*, 1993). This indicates heterogeneity of fructan metabolism between different members of the *Poaceae* family.

The variation seen in fructan structure between species is very large and the reason for this remains unknown. The different fructan structure could be a result of the evolution of FT and/or FEH in different plant species (Ritsema and Smeekens, 2003).

2.2.2 Accumulation and function of fructans

Besides their function in carbohydrate storage, fructans play other important roles in plants. Fructans are proposed to improve the response of the plants to drought, cold or pathogens (Hisano *et al.*, 2004a; Amiard *et al.*, 2003; Thomas and James, 1999; Pilon-Smits *et al.*, 1999; Yoshida *et al.*, 1998; Pilon-Smits *et al.*, 1995). Several studies have reported an accumulation of fructans, when the plants were exposed to drought (Amiard *et al.*, 2003; De Roover *et al.*, 2000; Thomas and James, 1999). However, it has not been shown that varieties with high fructan content have an increased resistance to drought compared to varieties with low fructan content. It is therefore important to establish whether this feature of fructans is present and can improve the drought resistance.

One study with different wheat cultivars showed that the content of fructans is positive associated with resistance to snow mould (Yoshida *et al.*, 1998). Furthermore, a correlation between cultivars with high fructan content and higher freezing tolerance was observed.

It is uncertain whether differences observed in fructan structures are a result of different needs in the plant or only reflect the conditions under which the plants were grown. It has been demonstrated that growth conditions influence the accumulation of fructans (Pavis *et al.*, 2001a; Sims *et al.*, 1993). The environmental conditions which increase the synthesis of fructans include: excision, illumination and cold. A study of excised leaves of *L. temulentum* showed that fructan concentration increased approx. 20 % between 6 to 26 hours after leaf excision (Sims *et al.*, 1993). The fructan content was depleted before the experiment by maintaining the plants under low irradiance. Ten hours past excision, fructans accumulated and after 48 hours all fructans normally present in *L. temulentum* were synthesised again. Cooling the roots combined with continuous illumination of leaves, stems and the expanding leaves of *L. perenne* also led to the accumulation of fructans (Pavis *et al.*, 2001a).

A few studies have looked for transcriptional regulation of fructan production by performing promoter analysis. In 2001, the first FT promoter was reported isolated from barley (pHv6-SFT) and using the reporter gene encoding β-glucuronidase behind the promoter and transform the construct into barley leaves, the promoter activity was demonstrated to be induced by sucrose and light (Nagaraj *et al.*, 2001). Subsequently, promoter sequences from *L. perenne* FTs have been reported (*LpFT4, Lp1-SST* and *LpFT1* (Chalmers *et al.*, 2005; Chalmers *et al.*, 2003; Lidgett *et al.*, 2002). The promoter sequence from *Lp1-SST* contains elements for light, stress and sucrose induction, while the *LpFT4* promoter sequence probably only contains elements for light and stress, whereas the *LpFT1* promoter predominately harbours light-regulated elements (Chalmers *et al.*, 2005).

Further investigations of FT promoters can provide important information on the regulation of fructan biosynthesis genes and fructan metabolism and will thereby support future improvement of the fructan content in grasses as well as other plant species. Especially, the regulation of FEHs, which degrades the fructans, would provide an efficient tool in regulation of fructan content and more knowledge about this gene family is highly valuable. Besides the gene regulation of FTs or FEHs, environmental factors, as temperature, light and growth period are important in regulation of accumulation of fructans.

2.2.3 Fructosyltransferases

FTs, together with invertases and FEHs, belong to glycoside hydrolase family 32, which are enzymes that catalyse the hydrolysis of glycosidic bonds and are key enzymes of carbohydrate metabolism (Henrissat and Davies, 1997; Henrissat, 1991).

The five classes of fructans identified in plants (figure 2.1 and table 2.1) are believed to be synthesised by the action of four FTs enzymatic activities: I) sucrose:sucrose 1-fructosyltransferase

(1-SST), II) fructan:fructan 1- fructosyltransferase (1-FFT), III) sucrose:fructan 6-fructosyl-transferase (6-SFT) and IV) fructan:fructan 6G- fructosyltransferase (6G-FFT) (Ritsema and Smeekens, 2003). Genes encoding for the four types of FTs have been cloned from different plant species and functionally analysed. No plant species has been demonstrated to contain all four FTs and none of the species analysed so far contains all five classes of fructans. Several other genes encoding putative FTs have been cloned but since they have not been functionally analysed it can not be excluded that these genes display the same enzyme activities as already described for the four functionally characterised FTs.

In 1995, the first FT (6-SFT) from barley was cloned (Sprenger *et al.*, 1995) and has been used for cloning of a variety of genes encoding FTs. Cloning of FT genes is a very challenging task as a result of a high GC content, especially in the 5' end. This feature makes it difficult to use primer base methods like RT-PCR and especially 5'RACE as method for cloning (Chalmers *et al.*, 2003; Luscher *et al.*, 2000). One way to overcome the difficulties is to screen libraries. However, because FT genes are very homologous to each other it is difficult to screen for one specific FT gene in a library. On the other hand the high sequence identities make it possible to screen a library from e.g. perennial ryegrass with a heterologous probe (Lasseur *et al.*, 2006). If primers or probes are designed in the UTR region, the cloning or screening becomes more specific.

To date, only the gene encoding 1-SST has been cloned from many different fructan-accumulating plants. A 6-SFT gene, however, could not be isolated from *Lolium* species as well as the fructan (Bifurcose) synthesises by 6-SFT (Pavis *et al.*, 2001b; Chatterton *et al.*, 1993).

The 1-SST enzyme is involved in the first step in the fructan metabolism irrespective of which class of fructans is produced. Based on several studies, it has been suggested that 1-SST is the rate-limiting enzyme of the fructan metabolism and that its regulation is crucial for the metabolism (Nagaraj *et al.*, 2004; Wiemken *et al.*, 1986; Edelman and Jefford, 1968).

Only a few full-length cDNA sequences encoding FT genes from *L. perenne* have been cloned and the majority of the cloned FTs are annotated based on sequence homology to other FTs in the databases. Recently, through functional characterisation, a gene encoding a FT, which previously was annotated to be a 1-SST based on sequence homology, was demonstrated to display different enzymatic characters when it was heterologous expressed in *Pichia pastoris* (Lasseur *et al.*, 2006). Besides a minor 1-SST, also 6G-FFT and 1-FFT activity was measured. Since the 1-SST activity was only present in a low amount and a gene encoding 1-SST from *L. perenne* has been cloned and functionally analysed, it was concluded that this new gene encodes for an enzyme displaying only 6G-FFT and 1-FFT activity (Lasseur *et al.*, 2006; Chalmers *et al.*, 2003). Triple activities has previously been demonstrated, however, this feature can not be excluded to be a result of the expression in *P. pastoris* (Hochstrasser *et al.*, 1998).

Phylogenetic analysis of the deduced amino acids from all isolated genes encoding FTs from *Lolium* compared to functionally analysed FT from other monocots indicates that several of the genes probably code for similar enzyme activities (figure 2.5).

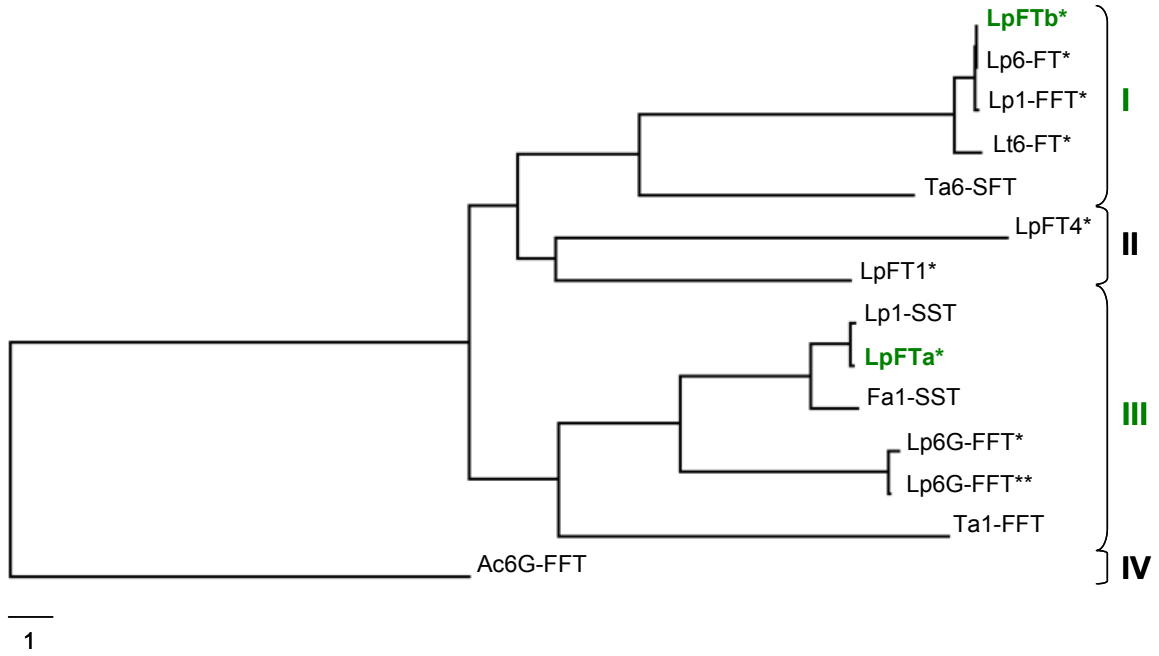

Figure 2.5 Phylogenetic dendrogram constructed from deduced amino acid sequences of full length fructosyltransferases isolated from *Lolium perenne* and functional analysed fructosyltransferases from selected monocots.
Their respective accession numbers are: *LpFTb*, DQ408727; *Lp6-FT*, AF494041; *Lp1-FFT*, AB186920; *L.temulentum 6-FT*, AJ532550; *Triticum aestivum 6-SFT*, AB029887; *LpFT4*, DQ073970; *LpFT1*, AF481763; *Lp1-SST*, AY245431; *LpFTa*, DQ408726; *Festuca arundinacea Fa1-SST*, AJ297369, *Lp6G-FFT*, AB125218; *Lp6G-FFT*, AF492836; *T. aestivum Ta1-FFT*, AB088409 and *Allium cepa Ac6G-FFT*, Y07838. FTs marked with green are isolated during the present PhD project. *: non-functional characterisation or ** recently renamed from Lp1-SST to Lp6G-FFT based on functional characterisation. The scale bar indicates a distance value on 1.

The amino acids sequences illustrated in the phylogenetic tree can be grouped in four groups (figure 2.4). One of the genes encoding a FT (*LpFTb*), which has been cloned during the current PhD project, shares high sequence identifies to other uncharacterised FTs from *L. perenne* (group I). They do all share highest sequence identities to a characterised FT from wheat which has been demonstrated to have 6-SFT activity (Kawakami and Yoshida, 2002). Based on the high sequence identity (96-99 %) is likely that *LpFTb* is identical to *Lp6-FT*. However, the enzyme function remains to be investigated from both genes.

Recently, *Lp1-FFT* was cloned (accession number AB186920) (Hisano *et al.*, 2004b) and it was suggested that *Lp1-FFT* encodes a 1-FFT (Chalmers *et al*., 2005). However, the sequence from *Lp1-FFT* shares higher sequence identities to the other FTs in group I and did not group with *Ta1-FFT*. The statement that *Lp1-FFT* encodes truly 1-FFT activity only based on sequence homologies seems therefore at least doubtful.

The second gene isolated during the present PhD project, *LpFTa*, is tightly clustered with two functionally analysed FTs with 1-SST activity (group III). Since *LpFTa* display 99 % sequence identity to *Lp1-SST* it is probably the same gene, which has been cloned.

Since several FTs have been reported to possess dual or even triple enzymatic activities depending on the substrate and/or the concentration of the substrate, functional characterisation of the enzyme activity is very important. A number of examples of multiple activities have been reported and recently a gene encoding a FT was cloned and through functional analysis, it was demonstrate to display 6G-FFT, 1-FFT and 1-SST activities when expressed in *P. pastoris* (Lasseur et al., 2006). In addition to the 6-SFT activity, 6-SFT from barley also display 1-SST and invertase activity (Hochstrasser *et al.*, 1998), 6G-FFT from onion also displays 1-FFT-like activity (Ritsema *et al.*, 2003; Vijn *et al.*, 1997) and 1-FFT from chicory can degrade fructans in the present of sucrose and thereby display FEHs activity (Van den Ende *et al.*, 1996).

2.2.4 Fructan exohydrolases

Fructans are primarily degraded by FEH which catalyze the β(2,1) (1-FEH) and/or β(2,6) (6-FEH) cleavage of linked fructose units. In general, fructans with low DP values are degraded faster than fructans with high DP values. Some FEHs degrade both fructans with β(2,1) or β(2,6) linkages (Kawakami *et al.*, 2005; Bonnett and Simpson, 1995). A special FEH with substrate specificity against the levan fructan, 6-kestose, was therefore named 6-kestosidase (6-KES) (Van den Ende *et al.*, 2005).

Since *Lolium* contain fructans with both β(2,1) and 2,6) linkages, it is expected that both 1-FEH and 6-FEH activities must be present in this species. From *L. rigidum*, five different FEHs have been purified and three of them showed higher hydrolysis of β(2,6)-linked fructans than β(2,1)-linked (Bonnett and Simpson, 1995). This is in agreement with the fact that *Lolium* predominantly contains fructans with β(2,6)-linkages. In *L. perenne*, purified 6-FEH activity predominantly degraded fructans with β(2,6)-linkages (Marx *et al.*, 1997). This enzyme can hydrolyse fructans with β(2,6) linkages at a rate five times higher than fructans with β(2,1) linkages.

Only one full-length and one partial gene encoding putative FEHs have been cloned from *L. perenne* but are not enzymatically characterised yet (Chalmers *et al.*, 2005; Lothier *et al.*, 2004). The putative FEH clones from *L. perenne* inclusive the six putative FEHs isolated during the present PhD project are aligned to other FEHs from other monocots (figure 2.6).

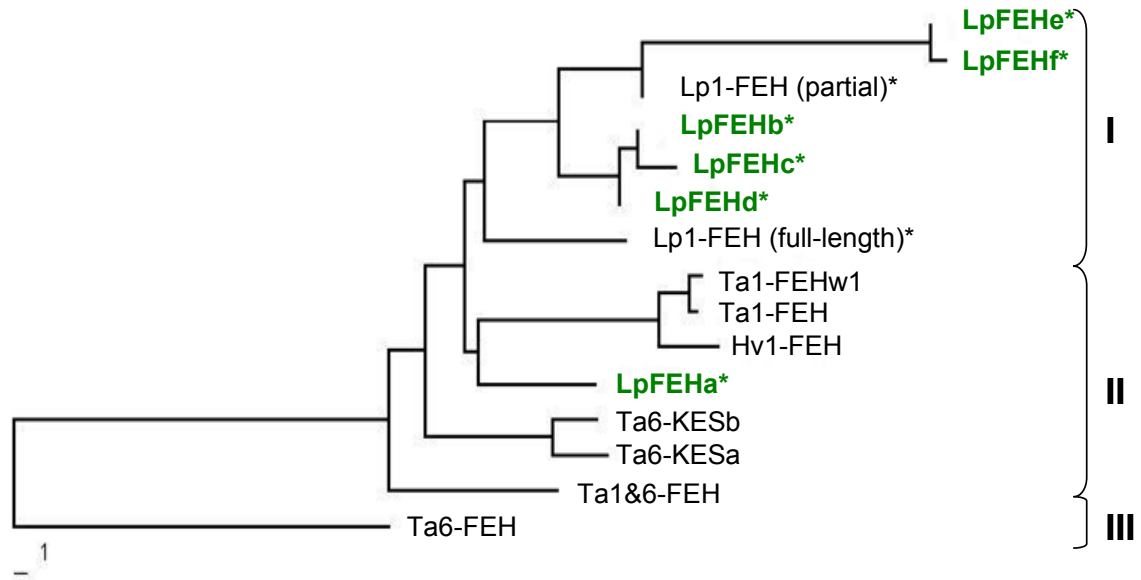

Figure 2.6 Phylogenetic dendrogram constructed from deduced amino acid sequences of fructan exohydrolase isolated from *Lolim perenne* and functional analysed fructan exohydrolases from selected monocots. Their respective accession numbers are: *LpFEH* (partial), AY693396; *Lp1-FEH* (full-length), DQ073968; *Triticum aestivum Ta1-FEHw1*, AJ516025; *Ta1-FEH*, AJ508387; *Hordeum vulgare Hv1-FEH*, AJ605333; *Ta6-KESb*, AB089271; *Ta6-KESa*, AB089270; *Ta6&1-FEH*, AB089269 and Ta6-FEH, AM75205. Names marked in green are isolated during the present PhD project and has no accession numbers. The scale bar indicates a distance value on 1.

Since the characterisation of the cloned FEH from *L. perenne* has not yet been performed, it is not certain whether these FEHs have 1-FEH activity, 6-FEH activity or activities against both fructan linkages. However, the phylogenetic analysis indicates that all the putative FEHs display higher sequence identities to 1-FEH than 6-KES or 6-FEH from wheat, which indicates all the genes encoding putative FEHs may display 1-FEH activity. As the gene(s) encoding the FT(s) responsible for the synthesis of levan neoseries has still not been cloned yet, also the gene(s) encoding FEH(s) lack to be identified. However 6-FEH activity has been found in *L. perenne* (Marx *et al.*, 1997).

Recently, an invertase from *L. perenne* was cloned and functionally analysed and the enzyme was demonstrated to exhibit invertase activity but could also degrade simple fructans and thereby demonstrating FEHs-like activities (Johnson *et al.*, 2003). This could indicate that besides FEHs, also invertases might play a role in degradation and/or metabolism of fructans.

2.2.5 Invertases

In plants, the utilisation of sucrose as a source for carbon and energy is depending of the cleavage of sucrose to fructose and glucose (Sturm, 1999). This cleavage is catalysed by sucrose synthase or invertases. Sucrose synthase is a glycosyl transferase, which in the presence of UDP converts sucrose into fructose and UDP-glucose. By contrast, invertase is a hydrolase which cleaves sucrose into fructose and glucose (Sturm and Tang, 1999).

Invertases exist in several isoforms with different subcellular locations and functions (figure 2.7).

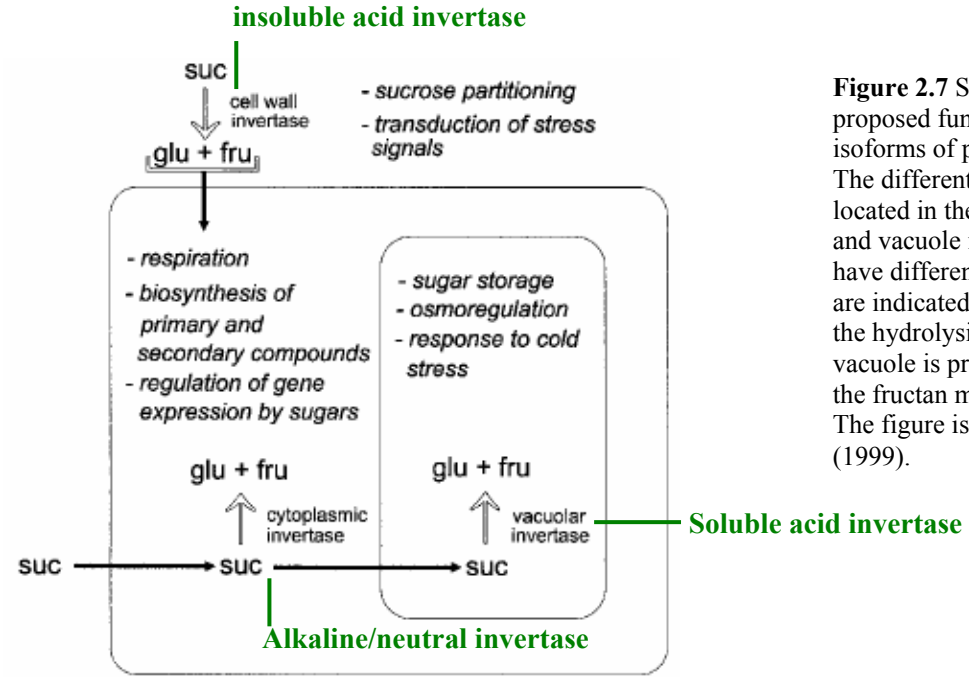

Figure 2.7 Subcellular locations and proposed functions of different isoforms of plant invertases.
The different invertase isoforms are located in the apoplast, cytoplasm and vacuole in the plant cells and have different functions (examples are indicated in italics). Especially the hydrolysis of sucrose in the vacuole is proposed to be critical for the fructan metabolism.
The figure is modified from Sturm (1999).

The invertases can be divided into two groups I) acid invertases with pH optima between 4-5.5 and II) alkaline/neutral invertase with pH optima in the range of 7-8. Acid invertases can be divided into two subgroups: the soluble and insoluble groups. The soluble acid invertases are believed to be present in the vacuole while the insoluble acid invertases are associated to the cell wall. The alkaline/neutral invertases are believed to be present in the cytoplasm. High activity of acid invertases are found in plant tissues with high demands for hexose, whereas alkaline/neutral invertases are involved in providing substrate for the TCA cycle in plant tissue where the activity of acid invertase or sucrase synthase are low.

Invertases, like fructosyltransferases, also display different activities depending on the substrate available and concentration. It has been reported that invertases can display 1-SST activity when the supply of sucrose is high (Obenland *et al.*, 1993). Invertases, besides fructan exohydrolases, are also known to be able to degrade small fructans with DP=3-7. Since invertases are ubiquitous in higher plants, they play, together with fructan exohydrolases, an unpredictable role in degradation of fructans. However, as it will be discussed in section 2.2.8, it is not evident that acid invertases in metabolic contact with sucrose used for fructan metabolism and might thereby not affecting the fructan metabolism either as fructan degrading enzymes or as competition for sucrose, since sucrose is the initiating substrate for fructan metabolism. In leaves of *L. temulentum*, it has been demonstrated that that acid invertases and sucrose are not in metabolic contact (Cairns and Gallagher, 2004b), arguing against a role of acid invertases in the competition for sucrose.

An invertase from *L. perenne* has been cloned and functional analysed (Johnson *et al.*, 2003). The enzyme was demonstrated to exhibit invertase activity against sucrose but could also degrade simple fructans demonstrating fructan exohydrolases-like activities. Opposite, 1-FEH has no

invertase activity since it cannot degrade sucrose due to its α(1,2) linkage present in sucrose (Van den Ende *et al.*, 2000).

2.2.6 Degradation of fructans

The enzymatic function of FEHs is to degrade fructans by remove external fructose molecules (exo-activity). However, to date no FEH which cut randomly within the fructan chain has been identified in plants but has been identified in bacteria and fungi (Van Damme and Derycke, 1983; Edelman and Jefford, 1968). Degradation of stored fructans in grasses can be triggered by many different environmental factors (Marx *et al.*, 1997). Defoliation, as a result of grazing or harvesting, is one example. After defoliation, fructans stored in stems are degraded to serve as a carbohydrate reserve for refoliation (Morvan *et al.*, 1997). The level of fructan in the stubbles after defoliation affects the production of the new leaf since the most of the photosynthesis material has been removed. It has been suggested that fructans serve as the sources for carbohydrate supply to sustain refoliation (Morvan-Bertrand *et al.*, 1999a). Detailed studies of fructan mobilisation after defoliation have been performed in *L. perenne* (Morvan-Bertrand *et al.*, 2001; Morvan-Bertrand *et al.*, 1999a; Morvan-Bertrand *et al.*, 1999b; Morvan *et al.*, 1997). It was observed that fructan depletion occurs in the stubbles during the first two days following defoliation. In addition, a variety with high fructan content displayed a faster rate of re-growth compared to a variety with low fructan content.

Results from a field study, where changes of fructan content in the roots of dandelion (*Taraxacum officinale*) as a result of seasonal changes were measured, indicated that when the plants are exposed to drought or cold, the levels of fructans with a degree of polymerisation (DP value) above 20 decreased significantly (Wilson and Kachman, 2001). Simultaneously, the amount of low-DP fructans (3-10) increased. This indicates that the plants responded to drought and cold stresses by degrading to high-DP fructans to smaller DP fructans.

It has been suggested that the sucrose concentration plays an important role in regulation of fructan degradation (Marx *et al.*, 1997). Sucrose concentrations in the shoots or stubble of different grasses were found to reach 50 mM, which is sufficient to inhibit fructan degradation by FEHs. Since a rapid decrease in the sucrose concentration in stems of *L. perenne* occurs after defoliation (Morvan *et al.*, 1997), it could explain the detected increased rate of fructan hydrolysis by FEH (Morvan-Bertrand *et al.*, 1999a). These results indicate that sucrose plays a role in regulation of FEHs. However, not all FEHs are inhibited by sucrose. Recently it was reported that a 6-FEH from wheat was not inhibited opposite to the 1-FEHs which are characterised to be inhibited by sucrose (Van Riet *et al.*, 2006; Van den Ende *et al.*, 2000).

Additional functions have been proposed for FEHs, since genes encoding enzymes with FEH activity have been cloned from the non-fructan accumulating plants; *Beta vulgaris* and *Arabidopsis thaliana* (De Coninck *et al.*, 2005; Van den Ende *et al.*, 2003). As no gene(s) encoding FT(s) have been purified from these species, it was suggested that FEH might play a role in protecting the plants against microbial infection by degrading the fructans, which are produced by pathogenic or symbiotic

bacteria (De Coninck *et al.*, 2005; Van den Ende *et al.*, 2003). Another possibility might be that FEHs degrade the fructans, which are synthesised as by-products of invertases in *A. thaliana* (De Coninck *et al.*, 2005) or simply that FEH degrades other sugar polymers than fructans.

It is also possible that FEHs might be involved in signal sensing since genes encoding FEHs have been isolated from non-fructan accumulating plants. The production of 1-kestose, 6-kestose and neokestose were detected, when excised and illuminated leaves from *A. thaliana* were incubated with sucrose (De Coninck *et al.*, 2005). These kestose might act as a signal and it is therefore possible that FEH are involved in removing the kestose signals (De Coninck *et al.*, 2005; Van den Ende *et al.*, 2004).

2.2.7 Fructan metabolism in *Lolium perenne*

To investigate the function of genes encoding FTs, transformation of FT genes into a non-fructan accumulating plant has been performed. It was demonstrated that the non-accumulating plants were able to synthesise the same fructans as the ones identified from the original plant where the genes have been isolated from. One example is artichoke where the 1-SST/1-FFT model, proposed by Edelman and Jefford in 1968, has been demonstrated to explain the pathways for fructan metabolism (Hellwege *et al.*, 2000; Edelman and Jefford, 1968). In onion, only two FTs; 1-SST and 6G-FFT have been identified and these enzymes are sufficient to produce the fructans identified in this plant (Ritsema *et al.*, 2003; Vijn *et al.*, 1998; Vijn *et al.*, 1997). However, in other plant species, different FTs are present and responsible for the synthesis of fructans.

Based on the fructan structures found in *L. perenne*, it has previously been proposed that at least four enzymes may be required to produce these three classes (inulin, inulin neoseries and levan neoseries) of fructans; 1-SST, 1-FFT, 6G-FFT and 6-XFT. However, cloning and functionally analysis of two FTs from *L. perenne* can only explain the synthesis of the inulin and inulin neoseries (Lasseur *et al.*, 2006; Chalmers *et al.*, 2003), whereas the FT (6-XST) responsible for the synthesis of levan neoseries remains to demonstrated. Therefore, only a hypothetical model for fructan metabolism in *L. perenne* can be proposed and the FT responsible for the levan production is marked 6-XFT (Figure 2.8).

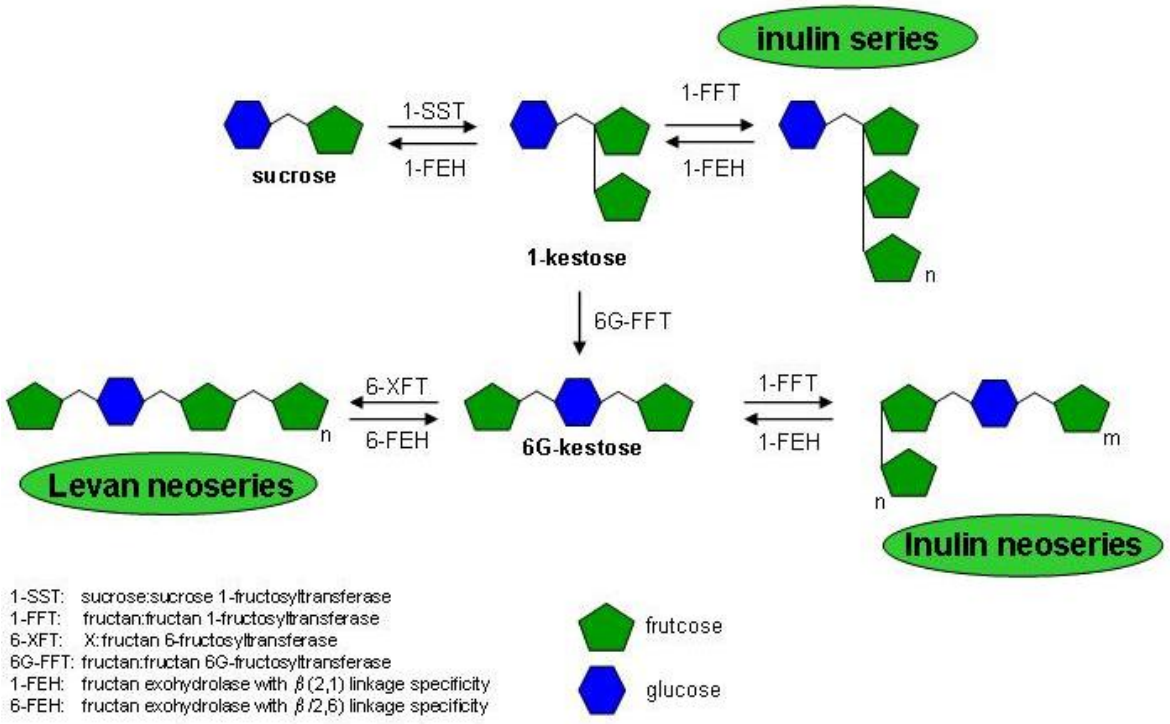

Figure 2.8 Model for fructan metabolism in *Lolium perenne* and the proposed enzyme activity of implicated FTs and FEHs.
Sucrose is the preliminary substrate and is utilised by 1-SST to produce 1-kestose. This substrate is then either elongated by 1-FFT to produce inulin series or used by 6G-FFT to produce 6G-kestose. 6G-kestose is elongated to produce inulin neoseries or levan neoseries by 1-FFT or 6-XFT, respectively. 1-FEH and 6-FEH can degrade β(2,1) and β(2,6)-linkages, respectively. m and n represent different number of fructose units.

It is still an unsolved question what determines the fructan chain length. It is obvious that the enzymatic activity of FTs plays an important role but also the activity of FEHs and/or invertases might define the final length of the fructan chain.

2.2.8 Localisation of fructan metabolism

Work by Cairns and coworkers (summarised by Cairns *et al*., 2000) have, based on calculations of sucrose concentrations in the vacuole, proposed the possibility that the fructan metabolism not only occur in the vacuoles. The substrate concentration of sucrose in the vacuole for metabolism of fructans should be at least 1.0 M. However, the sucrose concentration in the vacuolar sap of fructan accumulation mesophyll cells has only been measured to be 100-200 mM sucrose (Koroleva *et al*., 1997). This sucrose concentration would only result in an activity of 10-15% of the necessary rates to produce the amount of fructans found in plants.

Also presence of acid invertases in the vacuoles was investigated and the results obtained could only be explained by the hypothesis that invertases and sucrose are separated in different vesicles or vacuole types (Cairns and Gallagher, 2004a). Taken together with the fact that fructans as well as FTs and FEHs have been isolated from different compartments of the plant cell it is uncertain where and in which compartment the fructan metabolism takes place. Figure 2.9 illustrates the four possible hypothesis of the localisation of fructan metabolism based on the identification of fructans, FTs and/or FEHs.

The localisation of fructan metabolism in the vacuole is based on the isolation of FTs and FEHs from the vacuole (Wiemken *et al.*, 1986; Frehner *et al.*, 1984). If both FTs and FEHs are present in the vacuole, the plant may have a regulative system to adjust the activity of the enzymes. Another possibility could be that the enzymes display their different activities at different developmental stages.

In one model, fructan metabolism occurs in the vacuole where competition for the substrate sucrose occurs between invertases and FTs and is the model, which is general accepted (figure 2.9A).

Since it has been demonstrated that sucrose and acid invertases do not share metabolic contact (Cairns and Gallagher, 2004b), another hypothesis is proposed and illustrated in figure 2.9B. This hypothesis is supported by the presence of several types of vacuoles, which differ in function, content, size and shape (Vitale and Raikhel, 1999). When plant cells are formed by cell division in the shoot or root apical meristems, they contain hundreds of small pro-vacuoles (Taiz, 1992). During plant cell expansion, the pro-vacuoles fuse to form the central vacuole. There are at least two types of vacuoles: protein storage vacuoles and lytic vacuoles and they are probably merged in the mature plant cell (Vitale and Raikhel, 1999). It has also been proposed that fructans are synthesised in the cytoplasm and transported in vesicles to the vacuole since vesicle formation in storage cells of young tubes of artichoke have been localized in the cytoplasm (Kaeser, 1983).

Since fructans and FEHs have been isolated from the apoplast of winter oat (Livingston and Henson, 1998), it can not be excluded that at least the degradation of fructans occurs in the apoplast (figure 2.6C).

Several models for the location of the fructan metabolism have been proposed in figure 2.6 and the fact that different studies report evidence for all the proposed models, makes it difficult to predict which of them is correct. Multiple enzymatic functions of FTs impede to predict the precise function in fructan metabolism and correct location of the FTs. It seems bio-chemically unlikely that the plants should contain two sucrose competing enzymes in the same compartment (model A). It might be also possible that the plant can regulate the activity of acid invertase and FTs depending on the need or perhaps that two or more types of vacuoles exist. Finally, it is also possible that all models occur in the plant cell and that fructans can be degraded in the vacuole as well as in the apoplast, since FEHs have been located in both the vacuole as in the apoplast (Livingston and Henson, 1998; Wiemken *et al.*, 1986; Frehner *et al.*, 1984).

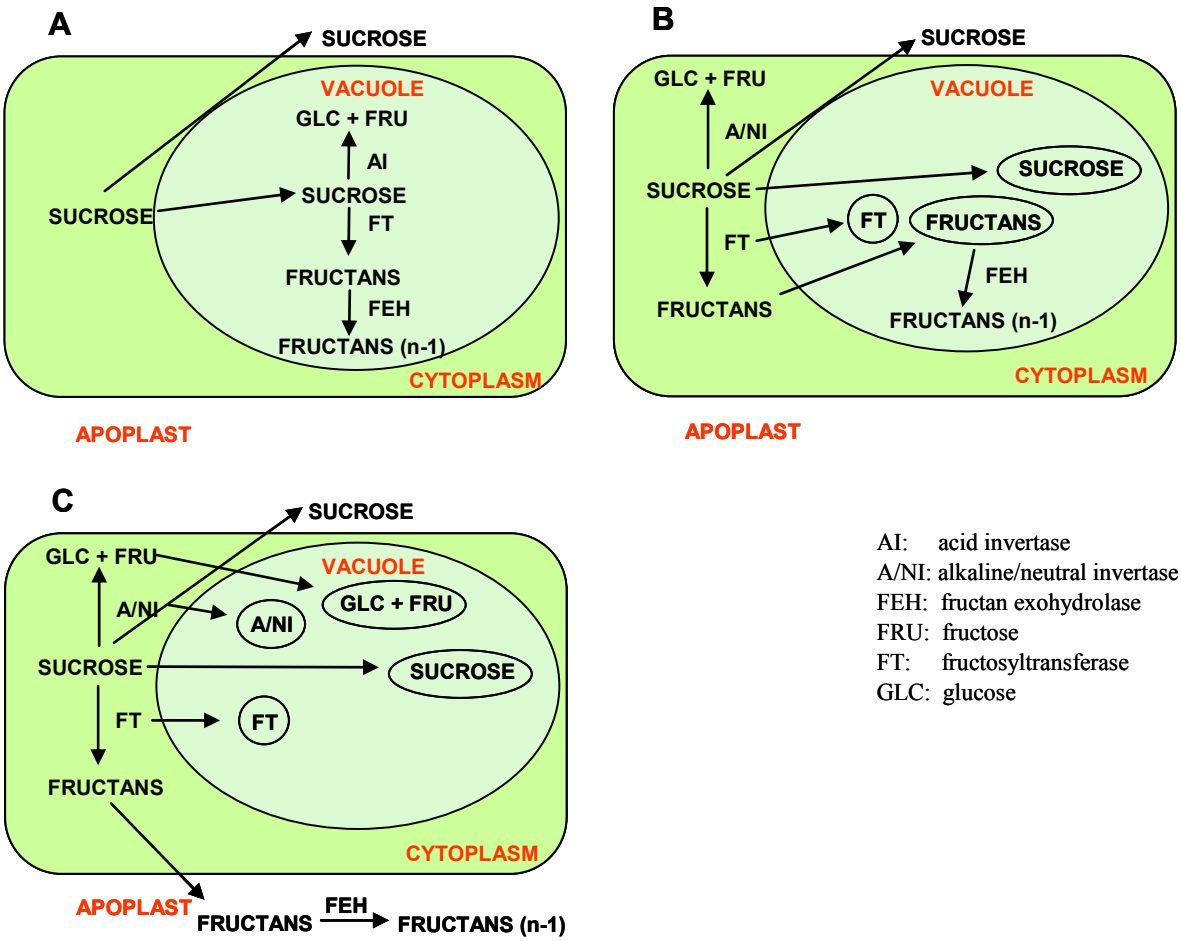

Figure 2.9 Four possible models of location of fructan metabolism in a plant cell. Photosynthetic activity in the chlorophyll is associated with synthesis of glyceraldehydes-3-phosphate, also called triose-P and is used for starch synthesis within the chloroplast or for sucrose synthesis in the cytosol (not illustrated). Sucrose is the preliminary substrate for fructan metabolism and four different metabolism pathways are illustrated (A-C). A: Sucrose is either transported to the apoplast or to the vacuole. In the vacuole, sucrose is either hydrolysed to glucose (GLC) and fructose (FRU) by the catalysis of acid invertase (AI) or used in fructan metabolism catalysed by FTs. Fructans can, however also be degraded in the vacuole under the action of FEHs. B: Sucrose is hydrolysed to GLC and FRU catalysed by alkaline/neutral invertase (A/NI) or used in fructan metabolism in the cytoplasm and later transported to the vacuole by vesicle formation. In the vacuole occurs the degradation of fructans. C: Sucrose is hydrolysed to GLC and FRU catalysed by alkaline/neutral invertase (A/NI) or used in fructan metabolism in the cytoplasm, whereas fructans are transported to the apoplast where fructan degradation occurs.

Since the work published by Wiemken *et al*. (1986) and Frehner *et al*. (1984) in the eighties no further investigations were published to confirm their results. Cairns and Gallagher made their investigations about the localisation of acid invertases in leafs 2004, but a real sorrow investigation on where and in which compartment(s) fructan synthesis and degradation takes place is still missing.

2.3 Transgenic approaches to improve fructan accumulation in plants

Plant transformation approaches designed to increase the fructan content began in the early 1990s after bacterial FT enzymes were purified and characterised. Levansucrase (*SacB*) from *Bacillus subtilis* is one example of the bacterial levan sucrase and it catalyses the synthesis of fructans with β(2,6)-linkages termed levan (Dedonder, 1966).

Non-fructan accumulating plants like maize, potato and tobacco were able to synthesise fructans ranging from 3 to 35 % after transformation with *SacB* (Ebskamp *et al.*, 1994; van der Meer *et al.*, 1994). Because it is believed that fructan metabolism in plants occurs in the vacuole of the plants and since bacteria do not contain vacuoles, a vacuolar targeting signal from yeast was fused to levansucrase sequence in all studies using *SacB* transformation of plants. However, none of the studies reported any evidence for the localisation of bacterial levansucrase in the vacuole and it has been reported subsequently that the levan produced in the transgenic lines was mainly localized outside the vacuole at the cell perimeter (Pilon-Smits *et al.*, 1996). The dislocation of fructans at other cellular locations may be the reason for the detrimental phenotypes seen in transgenic plants expressing bacterial levansucrase.

Introduction of fructan metabolism into potato plants strongly influenced starch metabolism (Pilon-Smits *et al.*, 1996). As no correlation between fructan and starch content could be observed, these results indicate that the decreased starch accumulation could be a result of other unknown factors and might not be directly related to fructan metabolism. In addition, it has been demonstrated in *L. temulentum* that starch metabolism is independent of fructan metabolism (Cairns *et al.*, 2002b).

Subsequently, the bacterial levansucrase has also been introduced into *L. multiflorum* and in this study, the vacuolar targeting signals from both yeast and potato were used to construct expression vectors for transformation (Ye *et al.*, 2001). Again, no investigation of localization was performed. Transgenic *L. multiflorum* harbouring the construct with either vacuolar targeting signals from yeast or potato were able to accumulate more fructans than the control lines. As for other transgenic studies with bacterial levansucrase, a changed phenotype was observed in the transgenic *L. multiflorum* and the plants were only two-thirds of the size of the control plants. However, the changed phenotype was first observed after two months of growth. This late effect could be a consequence of the low fructan content in the first month of growth, without a visible effect on the plants. Similarly, no changes in phenotypes could be observed in the transgenic tobacco plants with maximum 8 % fructan per g DW, which properly also is a result of the low fructan content (Ebskamp *et al.*, 1994). In another study with bacterial fructan accumulation, it was reported that, when fructan accumulation reached a certain threshold (7 % of dry weight), the growth of transgenic potato plants were affected supporting this hypothesis (Pilon-Smits *et al.*, 1996).

Since the first plant FT was cloned, plant FTs have been expressed in both fructan and non-fructan accumulating plants. Only one study has demonstrated that transformation of onion *6G-FFT* in

tobacco plants resulted in detection of 6G-FFT activity in the isolated vacuoles (Vijn *et al.*, 1997). However, it is also possible that the fructans were synthesised prior to entering the vacuole as illustrated in figure 2.6C.

In contrast to the expression of bacterial FTs, expression of plant FT in transgenic plants has not been reported to cause phenotypic changes. In a review of fructan biosynthesis in transgenic plants by Cairns (2003), it was suggested that the lack of phenotypic effect may be a result of the low fructan concentration in plants transformed with plant FT(s). However, the results presented in this thesis demonstrated that a fructan content up to 41 % fructan/g DW in transgenic *L. perenne* did not cause any phenotypic changes (figure 2.10) (chapter 3.1).

Figure 2.10 High fructan transgenic and a control line.
The transgenic line (left) is transformed with Ac1-SST and Ac6G-FFT in F6 genotype.
The control line (right) is a non-transgenic line regenerated from F6 callus.

It has been suggested that high fructan levels correlated to an increased tolerance against stress conditions such as drought and cold (Hisano *et al.*, 2004a; Pilon-Smits *et al.*, 1999; Pilon-Smits *et al.*, 1995). Bacterial levansucrase expression in tobacco plants increased the drought resistance and the drought tolerance was reported to be correlated to the level of levan produced (Pilon-Smits *et al.*, 1995). The study did, however, only report data from one transgenic line, so the conclusion must be drawn based on the comparison of wild type and this particular transgenic line. Similar observations were obtained using the same transgenic approach in sugar beets (Pilon-Smits *et al.*, 1999). This study demonstrated that the transgenic line with highest levan content also show the highest productivity under drought (Pilon-Smits *et al.*, 1999).

The ability of fructans to improve the plants response to cold has been investigated using transgenic plants expressing 1-SST or 6-SFT from wheat and measured with a electrolyte leakage assay (Hisano *et al.*, 2004a). The results demonstrated a negative correlation between fructan content and percent electrolyte leakage. Since cooling as well as dehydration causes phase changes in the lipid components of the membranes, it is possible that the interaction of fructans with the lipids increased the resistance to cold temperature or drought (Hincha *et al.*, 2002). As for the drought experiments, it has not been reported that varieties with a high fructan content have an increased resistance to cold than varieties with a low fructan content. It is therefore important to establish whether this feature of fructans is present and can improve the winter hardness of grasses which is an important character since high frost tolerance is correlated with strong spring growth.

3. Papers

3.1 Improved fructan accumulation in perennial ryegrass transformed with the onion fructosyltransferase genes 1-SST and 6G-FFT

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Submitted to Journal of Plant Physiology.

Summary

Carbohydrate limitation has been identified as a main reason for inefficient nitrogen use in ruminant animals, which feed mainly on fresh forage, hay and silage. This inefficiency results in suboptimal meat and milk productivity. One important molecular breeding strategy is to improve the nutritional value of ryegrass (*Lolium perenne*) by increasing the fructan content through expression of heterologous fructan biosynthetic genes. We have developed perennial ryegrass lines expressing sucrose:sucrose 1-fructosyltransferase and fructan:fructan 6G-fructosyltransferase genes from onion (*Allium cepa*) which exhibited up to 3-fold increased fructan content. Furthermore, the high fructan content was stable during the growth period whereas the fructan content in an elite variety, marketed as a high sugar variety, dropped rapidly after reaching its maximum and subsequently remained low.

Keywords: Fructans, heterologous transformation, *Lolium perenne*, fructosyltransferases, transcription levels.

Abbreviations: *1-SST*, sucrose:sucrose 1-fructosyltransferase; *6G-FFT*, fructan:fructan 6G-fructosyltransferase; 2,4-D, 2,4-dichlorophenoxyacetic acid; Ac, *Allium cepa*; CM, callus-induction medium; DP, degree of polymerisation; DW, dry weight; FW, fresh weight; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GOI, gene of interest; WSC, water-soluble carbohydrate.

Introduction

Fructans are polymers of fructose consisting of one sucrose molecule, with additional fructose molecules linked in β(2,1) or/and β(2,6) positions. In plants, five classes of fructans have been identified based on their structures: a) linear inulin with β(2,1) linkages, b) inulin neoseries with β(2,1) linkages linked to C1 of the fructose molecule and C6 of the glucose molecule, c) levan with β(2,6) linkage, d) levan neoseries with β(2,6) linkages linked to C1 of the fructose molecule and C6 of the glucose molecule and e) mixed levan with both β(2,1) and β(2,6) linkages (Ernst et al. 1998; Lüscher et al. 1996; Vijn et al. 1997). In *Lolium perenne* (perennial ryegrass), three out of the five classes of fructan have been identified; inulin, inulin neoseries and levan neoseries (Pavis et al. 2001b). Two genes encoding fructosyltransferases have been cloned and analysed functionally in *L. perenne* showing sucrose:sucrose 1-fructosyl-transferase (1-SST) and fructan:fructan 6G-fructosyltransferase/ fructan:fructan 1-fructosyl-transferase (6G-FFT/1-FFT) activity (Chalmers et al. 2003; Lasseur et al. 2006). These enzymes can explain the synthesis of fructans belonging to the inulin and inulin neoseries but not the synthesis of the levan neoserie which requires an enzyme which can link

the fructose molecule in β(2,6) position e.g. sucrose:fructan 6-fructosyl-transferase (6-SFT) or fructan:fructan 6-fructosyl-transferase (6-FFT) (Chalmers et al. 2005). Three genes, annotated 1-FFT, 6-FT and 6G-FFT have been cloned from *L. perenne* but have not been analysed functionally, so the enzyme activity is currently unknown (NCBI accession number AB125218, AB186920 and AF494041(Hisano et al. 2003; Hisano et al. 2004b; Lasseur et al. 2002), respectively).

Several models have been proposed to describe fructan biosynthesis. A general assumption is that four fructosyltrans-transferases are necessary for fructan production in higher plants; 1-SST, 1-FFT, 6-SFT and 6G-FFT (Koops and Jonker, 1996, Lüscher et al 1996, Van den Ende and Van Laere, 1996, Sprenger et al 1995, Shiomi, 1989, Wiemken et al., 1995 and Vijn et al., 1997). However, no plant species investigated so far has been demonstrated to contain all four genes encoding fructosyltransferases nor have the five classes of fructans been isolated from any single plant species. The highest number of different fructosyltransferases (1-SST, 1-FFT and 6-SFT), which have been cloned and characterised, are from wheat (Kawakami and Yoshida 2002; Kawakami and Yoshida 2005). In *e.g.* onions, it has been demonstrated that 1-SST and 6G-FFT are by themselves sufficient to synthesise the types of fructans naturally present in this plant, indicating that no other fructosyltransferases are required for fructan biosynthesis (Ritsema et al. 2003; Weyens et al. 2004).

Perennial ryegrass is one of the most important forage crops in temperate regions of the world and highly valued for dairy and sheep forage systems due to its high palatability and digestibility (Delagarde et al. 2000). However, the nutritional value of perennial ryegrass is lower than in other high quality fodders like maize silage. The lower nutritional value is caused by an inefficient utilisation of amino acids by micro-organisms in the rumen and is attributed to low carbohydrate levels in the grass. This inefficiency leads to an excess of ammonia which is released to the environment (Lee et al. 2003; Miller et al. 2001). One approach to avoid this inefficiency is to increase the carbohydrate content of the fodder, a strategy used in grass breeding programmes for several years (Marais et al. 2003), and the effect of carbohydrate composition has been investigated by feeding ruminants with high or low sugar diet. A recent study demonstrated that high sugar content in grass forage increased the nutritional value and thereby increased the milk production in dairy cows (Miller et al. 2001). As an additional desirable effect, a decline in the ammonia concentration released to the environment has also been measured where dairy cows were fed on a high sugar diet (Lee et al. 2003).

An alternative to increasing the sugar content in ryegrass by traditional breeding strategies is to use transgenic approaches. Increasing the fructan content is one possibility, and several transgenic approaches using a number of fructosyltransferases from bacteria or different plant species have been attempted with variable success (Caimi et al. 1996; Hellwege et al. 2000; Hisano et al. 2004a; Vijn et al. 1997; Weyens et al. 2004; Ye et al. 2001). The gene encoding bacterial levansucrase has been transformed into plants, but the transgenic lines were morphologically disordered (Caimi et al. 1996; Ye et al. 2001). Encouragingly, this negative effect was not reported when transgenic studies utilised plant fructosyltransferases (Hellwege et al. 2000; Hisano et al. 2004a; Vijn et al. 1997; Weyens et al. 2004).

In this investigation, we transformed perennial ryegrass with the onion fructosyltransferases genes *1-SST* (Vijn et al. 1998) and *6G-FFT* (Vijn et al. 1997). We obtained transgenic ryegrass lines with up to 3-fold higher fructan level compared to control lines and the fructan content remained high after reaching its maximum. Furthermore, TLC analysis indicated a different fructan finger print arguing that new fructans with higher degree of polymerisation (DP) were synthesised in transgenic lines compared to control lines.

Materials and Methods

Growth conditions

The plants used were initiated from a single tiller to synchronise the growth, and were allowed to grow for three to seven months under greenhouse conditions (22 ºC for 16 h/20 ºC for 8 h). The transgenic and control lines were placed randomly in the growth area during the growth period. All samples were harvested at noon and at the same height (6 cm above ground level) to avoid diurnal variation in water-soluble carbohydrate (WCS) content. The selected harvest height was chosen since it is the optimal height for re-growth (Fulkerson and Donaghy 2001).

Transformation of Lolium perenne

The coding regions of *Ac1-SST* (accession no AJ006066) (Vijn et al. 1998) and *Ac6G-FFT* (accession no Y07838) (Vijn et al. 1997) were amplified with following primers; K41-fwd 5'-ATGGAATCCAGAGATATCGAG-3, K41-rev 5'-CCCGGGTCAAGGAGCTGGAAATCCGG GG AATGG-3', K42-fwd 5'-TGGATGCTCAGG ATATTGAGTCCCGTCAC-3' and K42-rev 5'-CCCGGGTTAAAAATGATAAAAATCATTGTA

AGTGGAGTTCATTTGCC-3'. PCR reactions (50 µl) contained 0.1 µg DNA, 5 units of PFU polymerase (Stratagene, USA), 1x PFU reaction buffer, 5 nmol dNTP and 1 pmol of each forward and reverse primer. Amplification was achieved by denaturation at 95ºC for 2 min followed by 35 cycles of 95ºC for 15 sec, 50-60ºC for 1 min and 72ºC for 5 min depending on the optimal temperature of the primers. The obtained PCR products were restricted with 10 units of *Xma*I (Invitrogen, USA).

The vector L42 (Petersen et al. 2006) was restricted with *Nco*I, overheads removed using T4 DNA polymerase according to the manufacturers' protocol (Invitrogen, USA) and restricted with *Xma*I (Invitrogen, USA). The PCR products were ligated into L42 to create plasmids designated K41 and K42, respectively (figure 3.1). These plasmids were co-transformed into perennial ryegrass together with pAHC20 (B24) or pJFNPTII (J34) (Altpeter et al. 2000; Christensen and Quail 1996). B24 harbours the *bar* gene, which confers resistance to the herbicide Basta® whereas J34 harbours the *nptII* gene, which confers resistance to kanamycin. The obtained fragments were sequenced in both directions as control of PCR errors at MWG-Biotech (Germany) and the DNA sequence obtained was analysed by DNASTAR, version 5.05 (Lasergene, USA).

Embryogenic callus induced from meristems of perennial ryegrass (*Lolium perenne* L, genotype N2 and F6, DLF-TRIFOLIUM) was prepared as described by Petersen *et al*. (2006) with a few modifications: K41, K42 and the selection marker (B24 or J34) were transformed by a molar ratio of 1:1:1. K41, K42 and B24 were transformed into N2 callus whereas K41, K42 and J34 were transformed into F6 callus. After several rounds of selection at three weeks interval, putative transgenic N2 callus were transferred to hormone-free medium (MS medium containing 3% (w/v) sucrose and 0.2 mg/l kinetin) supplemented with 3 mg/l bialaphos and for selection of F6 callus, the MS-medium contained 50 mg l-1 paromomycin (Duchefa, The Netherlands). During antibiotic selection, the concentration of paromomycin was increased to 100 mg l-1. Emerging plantlets were transferred to containers containing MS-medium at half strength for further development and root formation. Finally, lines were transferred to soil and grown to maturity under greenhouse conditions.

Analysis of water-soluble carbohydrate content
Samples were dried in a Heto FD3 freeze dryer (Heto-Holten, Denmark) for 48 hrs, ground and passed through 0.5 mm filter in a MF 10 BASIS mill (IKA Werke, Germany). The percentage dry weight was determined by measuring the weight before and after the freeze drying.

500 mg material was extracted in 25 ml 100 mM Acetate buffer (pH=5.0) at 65ºC for 60 min. A 2 ml sample was hydrolysed by adding 2 ml 74 mM H_2SO_4 at 80ºC for 70 min. Assay mixture was prepared by adding 1 ml triethanolamine buffer, pH=7.6 (14 v/w % triethanolamine hydrochloride and 0.25 v/w % magnesium sulphate, 7 H_2O), 0.1 ml ATP solution (4.55 v/w % ATP disodium salt hydrate and 5 v/w % sodium hydrogen carbonate), 0.1 ml 0.943 v/w % β-NADH phosphate disodium salt and 0.1 ml extracted or hydrolysed sample to a total volume of 3.2 ml. The glucose content was

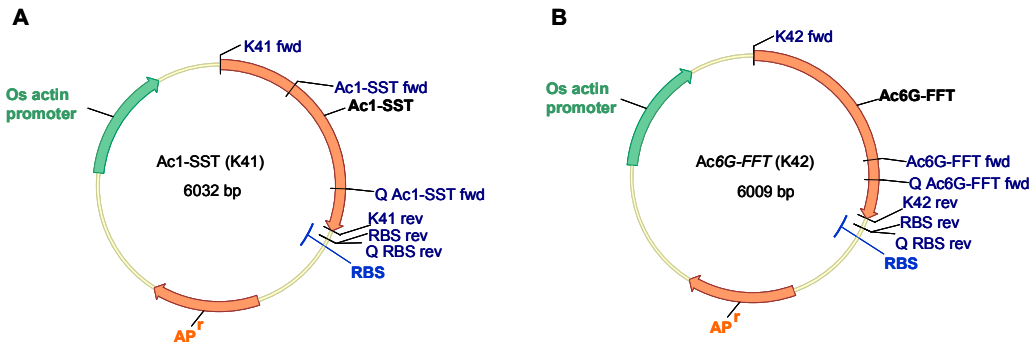

Figure 3.1 Plasmid maps of K41 and K42.
A: Ac1-SST (K41) and B: Ac6G-FFT (K42). In both maps primers used for cloning, PCR, RT-PCR and Q-PCR are marked.

determined by measuring NADPH formation (Hitachi U-1100 Spectrophotometer, Japan) at 340 nm after incubation with 6.8 units of hexokinase (HK)/3.4 units of glucose-6-phosphate dehydrogenase (G6-PDH) (Roche, Germany) for 15 min (Cairns 1987; Knudsen 1997; Larsson and Bengtsson 1983). Total hexose (glucose plus fructose) was determined by adding 0.7 units of phosphor glucose isomerase (PGI, Roche, Germany) to the previous reaction and formation of NADPH was measured after 15 min. The content of glucose and fructose in the extraction sample is defined as glucose and fructose, whereas content of glucose and fructose after hydrolysis is defined as sucrose and fructan. Each sample was repeated twice and as reference for the extraction, hydrolysis and enzymes used for measurements, a Soya sample was included.

DNA isolation and PCR analysis
Genomic DNA was isolated from leaves of primary transformants (T0 generation) using DNeasy 96 plant kit (Qiagen; Germany) and the presence of the GOI (*Ac1-SST* and/or *Ac6G-FFT*) was determined by PCR. The primers used for confirmation of the genomic integration of the transgenic DNA: *Ac1-SST*-fwd (5'-AACGGCAAC CCCATCCTCAT-3), *Ac6G-FFT*-fwd (5'-TGAGG CCACAATAG AAGCAGATG-3), RBS-rev (5'-TATCTGGGAACTACTCACACA-3).

PCR reactions (10 µl) contained 0.5 µg gDNA, 1.4 U of Expand DNA polymerase, 1x Expand reaction buffer 2 (Roche, Germany), 5 nmol dNTP and 0.5 pmol of each forward and reverse primer. Amplification was achieved by denaturation at 95ºC for 2 min followed by 35 cycles of 95ºC for 15 sec, 58-62ºC for 30 sec and 72ºC for 1-2 min depending on the optimal temperature of the primers and length of the products, respectively.

DNA gel blot analysis
Genomic DNA was isolated as described above and 20 µg was restricted overnight with 100 units of *Hpa*I and *Pvu*II (Invitrogen, USA). As controls plasmids K41 and K42 were restricted with 10 units of *Hpa*I and *Pvu*II releasing the part of the cassette containing the promoter, GOI and terminator. The restricted DNA samples were fractionated on a 0.8% agarose gel (Invitrogen, USA) and blotted onto Amersham Hybond N membrane according to Sambrook *et al*. (1989). The membrane was hybridised with a 420 and 480 bp gene specific DNA fragment from *Ac1-SST* or *Ac6G-FFT*, respectively. The probes were labeled with ^{32}PdCTP (GE Healthcare, UK) using the random primer method (Megaprime, GE Healthcare, UK). Prehybridisation, hybridisation and the subsequent washing steps were performed as described (Sambrook et al. 1989). Signals were detected by exposing the membrane to autoradiography films (Biomax, Kodak) for two to seven days at -80 ºC using a Biomax Transscreen HE intensifying screen (Kodak).

RNA isolation, RT-PCR and Q-PCR analysis
Purified mRNA was isolated from leaves of primary transformants (T0) with Dynabeads (DYNAL, Norway) and single strand cDNA was transcribed using Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen, USA).

Determination of measurable transcription from *Ac1-SST* and *Ac6G-FFT* was performed by RT-PCR using the same primers as for determination of genomic integration of the constructs. RT-PCR reactions of 10 µl contained 0.5 µg cDNA, 0,25 U of Amplicon DNA polymerase, 1x standard reaction buffer 2 (Invitrogen, USA), 5 nmol dNTP and 0.5 pmol of each forward and reverse primer. Amplification was achieved by denaturation at 94ºC for 2 min followed by 35 cycles of 94ºC for 15 sec, 58-62ºC for 30 sec and 72ºC for 1-2 min depending on the optimal temperature of the primers and length of the products, respectively.

An aliquot of 1/25 of the RT reactions was applied for PCR amplifications performed in a quantitative Rotorgene 2000 system (Corbett Research, Australia) as described by (Petersen et al. 2004). The primers were: Q*Ac1-SST*-fwd (5'-GTAGGAATGTTGATGGTGG T-3'), Q*Ac6G-FFT*-fwd (5'-CGCCTTACGCACTCACT-3'), Q*RBS*-rev (5'-ACAAGGTGGGAGACATCA TCGA-3'), Q*LpFTa*-fwd (5'-CGATGGGCGG GAGGA-3'), Q*LpFTa*-rev (5'-GGTTTCATCT ACAAGTCGTCG T-3'), Q*LpFTb*-fwd (5'-GG AAATTCCTGCAACTCGA-3') and Q*LpFTb*-rev (5'-CTGGGGCGGTC CTGCT-3'). Amplification was achieved by denaturation at 95ºC for 15 min followed by 35 cycles of 95ºC for 15 sec, 55-58ºC for 30 sec and 72ºC for 30 sec followed by a melting point at 80-88ºC depending on the primers. The samples were analysed single or in triplicates and after each performance of Q-PCR, the PCR products were verified for correct size and specificity on a 2% agarose gels (Invitrogen, USA) with 2 mg /ml Ethidium Bromide (Invitrogen, USA).

Thin Layer Chromatography
The material used for thin layer chromatography analysis was extracted as described under water-soluble carbohydrate analysis. Aliquots (1 ml) were evaporated to dryness in a Maxi Dry Lyo speed vacuum centrifuge (Heto-Holten, Denmark)

and dissolved in 50 µl water. Ten µl were spotted onto 20 x 20 cm aluminium sheets Silica Gel 60 F_{254} and the chromatograms were for developed twice at room temperature in a mixture of butan-1-ol:propan-1-ol:water in a ratio of 3:12:4 v/v (Sigma-Aldrich, USA). First run was performed over night, where after the plate was dried with a hairdryer before it was developed again for 8 hrs. The chromatograms were made perceptible by spraying with a fructose specific reagent (Wise et al. 1955) and heated for 5 min at 80ºC.

A standard was prepared by adding equal amount of 1 % (v/w) fructose, sucrose, 1-kestose and 1,1-kestotetraose, respectively and run as a mixture. As an alternative standard 2 g of fresh Danish wild type Chicory was extracted as described under WCS analysis. From these standards, 6 µl were spotted on the chromatograms.

Cloning and molecular analyses

Total RNA was isolated from the aerial part of vegetative perennial ryegrass (*Lolium perenne* L, clone F6, DLF TRIFOLIUM) or from plants subjected to three month vernalisation (~5°C). mRNA was isolated as described above and used to generate a cDNA library using the ZAP-cDNA synthesis kit (Stratagene) according to the manufacturer's instructions.

Screening of the libraries was performed at 65ºC with ^{32}P-dCTP labelled (GE Healthcare, UK) probes and positives clones were isolated according to the Stratagene protocol. Sequencing of the clones in both directions was performed at MWG-Biotech (Germany) and the DNA sequence obtained was analysed by DNASTAR, version 5.05 (Lasergene, USA).

The library made of vernalised plants was screened with a 900 bp fragment of the *6G-FFT* from onion (accession no Y07838) (Vijn et al. 1997) resulting in cloning of a full-length clone designated *LpFTa* (accession no DQ408726). An 831 bp fragment of artichoke *1-SST* (accession no Y09662) was used as probe for screening of the library made of the aerial part of vegetative plants, resulting in the cloning of a full-length cDNA designated *LpFTb* (accession no DQ408727).

Neighbour-joining analysis was performed using ClustalW from Megalign, version 5.05 (Lasergene, USA). Deduced amino acid sequences from plant fructosyltransferase genes used for the phylogenetic tree were applied from the NCBI GenBank database.

Results

Transformation of perennial ryegrass with heterologous fructosyltransferases results in a 3-fold increased fructan content

Around 70 Basta® or kanamycin resistant T0 transgenic ryegrass lines were obtained by co-transformation of onion *1-SST* (K41) and *6G-FFT* (K42) genes together with either *bar* or *nptII* gene in callus of either N2 or F6 genotype. None of the transgenic lines displayed any visual morphological changes when compared to control lines. Around 50% of the transgenic lines from both genotypes contained at least K41 or K42 and half of those transgenic lines also transcribed the introduced gene(s) (table 3.1).

All the transgenic lines, with detectable transcription of at least one or both genes (K41 and/or K42), were analysed for WSC content and several transgenic lines exhibited significant increased fructan content and up to 3-fold higher fructan levels compared to control lines. The highest content measured was 41 % fructan per g DW and was obtained in the N2 genotype (figure 3.2A). The highest content measured in a transgenic line with F6 genotype was 21 % fructan per g DW (figure 3.2B). In general, higher fructan contents were measured in transgenic lines of N2 genotype compared to F6 genotype, but as a result of lower transformation efficiency for N2 compared to F6, the N2 genotype was replaced by F6 genotype during the process of transformation of *Ac1-SST* and *Ac6G-FFT*.

Table 3.1 Transgenic lines transformed with *Ac1-SST*, *Ac6G-FFT* and a selection plasmid by co-bombardment.

	N2 genotype	F6 genotype
Number of resistance transgenic lines	28	41
Number of transgenic lines with presence of at least one GOI[a]	16 (15)	23 (19)
Number of transgenic lines with transcription of at least one GOI[b]	9 (7)	12 (12)
Number of transgenic lines with high fructan content[c]	9	1

a: The number in brackets represents the number of transgenic lines with presence of both GOI. b: The number in brackets represents the number of transgenic lines with transcription of both GOI. c: The fructan content in the transgenic lines is compared to the fructan content in control lines at 15 % per g DW.

In contrast to the measured increase in fructan content, no significant differences in glucose, fructose or sucrose contents were measured in the transgenic lines compared to the control lines irrespective of the genotype (figure 3.2).

Out of the 21 transgenic lines, which were positive for measurable transcription of at least one of the introduced genes, 10 transgenic lines showed a fructan content significantly higher than the content measured in control lines (table 3.1 and figure 3.2). Nine out of the ten transgenic lines were obtained in the N2 genotype, whereas transformation of the F6 genotype only result in one transgenic line with improved fructan content.

In order to exclude a possible effect on fructan content caused simply through the transformation process, 23 control lines have been analysed for fructan content and none of them contained more than 15 % fructan per g DW after five months of growth in the greenhouse (figure 3.2C). The group of control lines consisted of 12 transgenic lines with no detectable transcription of the introduced genes (T1-T12), eight untransformed elite varieties (E1-E8) and three lines regenerated from callus, but not bombarded with control plasmid or gene of interest (B1-B3). The content of glucose, fructose and sucrose was not different from the content in the transgenic lines (data not shown).

Measuring the biomass, as percentage dry weight after drying the samples at 60°C for 48 hrs, the transgenic lines with significantly higher fructan content consisted of 30-35 % dry weight, whereas the control lines and the transgenic lines with similar or even lower fructan content contained an average dry weight of 25% (data not shown).

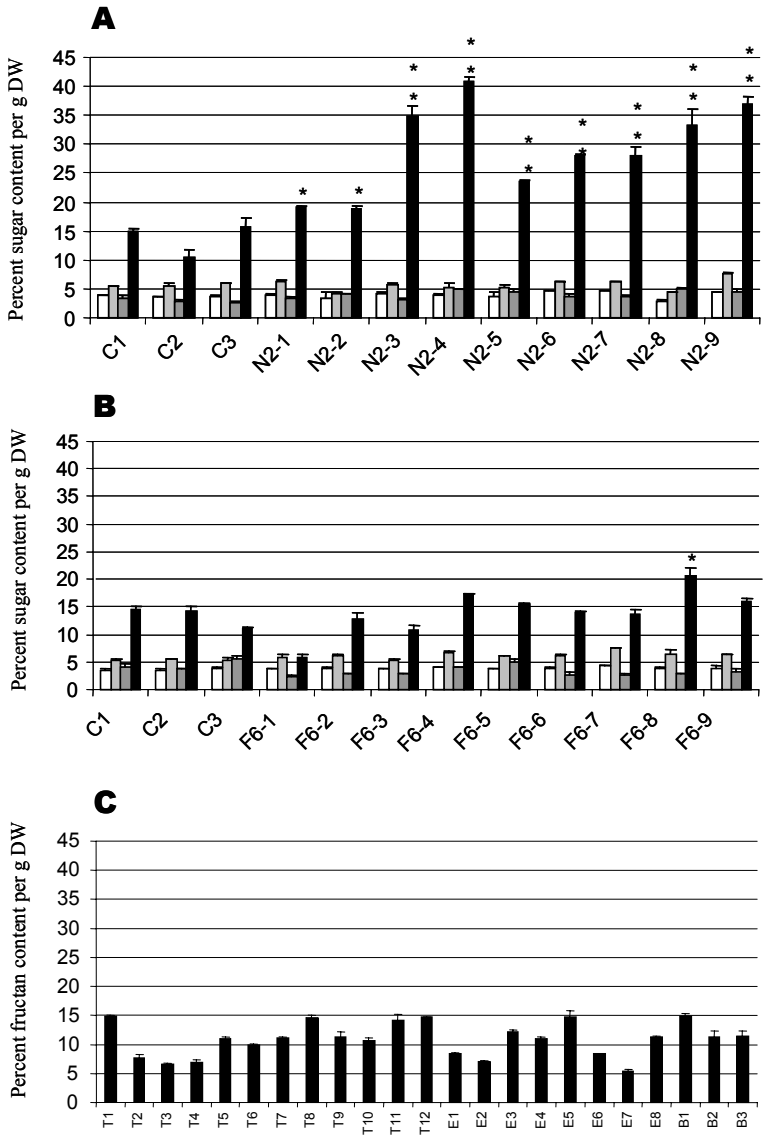

Figure 3.2 Sugar content in control and transgenic perennial ryegrass lines.
The amount of sugar in N2 genotype (A), F6 genotype (B) and the amount of fructan in control lines (C) was measured by enzymatic method. The sugar content is represented by glucose (□), fructose (□), sucrose (□) and fructan (■). C1 and C2 are non-transgenic lines (N2 (panel A) and F6 (panel B) genotypes), whereas C3 is transformed with a control plasmid from each transformation. N2-1 (+/+), N2-2 (+/+), N2-3 (+/+), N2-4 (+/+), N2-5 (+/+), N2-6 (+/+), N2-7 (+/+), N2-8 (+/-) and N2-9 (+/-) are transgenic lines with N2 genotype. F6-1 (+/+), F6-2 (+/+), F6-3 (+/+), F6-4 (+/-), F6-5 (+/-), F6-6 (+/+), F6-7 (+/+), F6-8 (+/-) and F6-9 (+/-) are transgenic lines with F6 genotype. +/+ illustrates measurable transcription of *Ac1-SST* and Ac*6G-FFT* and +/- illustrates measurable transcription of only *Ac1-SST*. All lines were harvested 6 cm above the ground. Data from transgenic lines were analysed for significant differences from control lines using *t* test. Values are the mean of duplicate extractions ± SE. Where no error bar is included, the error was smaller than the symbol. *Asterisks* indicate; *P<0.025; **P<0.005.

The fructan pattern was analysed by thin layer chromatography in order to identify differences in fructan patterns in control and transgenic lines with different fructan content attributable to the transgenic onion fructosyltransferases. Both the control and the transgenic lines contain fructans with such high DP values that they did not migrated into the matrix and could not be separated in the used experimental setup, which is indicated by the strong labelling of the spotting site.

Nevertheless, the transgenic lines with high fructan content contained different fructan pattern (figure 3.3, lane 6-10) which could not be observed in the control lines (figure 3.3, lane 3-5). Some transgenic lines contained additional fructans with larger DP values (figure 3.3, lane 7-10) whereas other lacked fructans (figure 3.3, lane 6 and 10), which could be observed in the other transgenic lines and the control lines. This different fructan fingerprint is taken to indicate the action of the introduced heterologous fructosyltransferases.

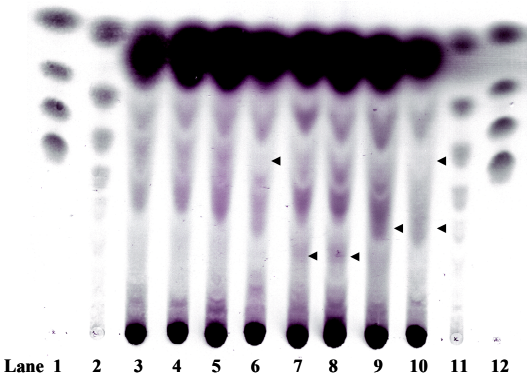

Figure 3.3 Thin Layer Chromatography of fructans in transgenic and control lines.
Comparison of structures of fructans from transgenic and control lines. Lane 1: standards, 2: Chicory, 3: C1 (N2 genotype), 4: C2 (F6 genotype), 5: C3 (transformed with a control plasmid in F6 genotype), 6: N2-1, 7: N2-2, 8: N2-3, 9: N2-4, 10: N2-5, 11: Chicory and 12: standards. C1 and C2 are non-transformed line and C3 is transformed with a control plasmid. N2-1 (+/+), N2-2 (+/+), N2-3 (+/+), N2-4 (+/+) and N2-5 (+/+) are transgenic lines with transcript of *Ac1-SST*/Ac*6G-FFT* (+/+). Markers DP1, DP2, DP3 and DP4 represent fructose, sucrose, 1-kestose and 1,1-kestotetraose, respectively and are applied at approximately 50 µg each and chicory is applied at approximately 40 µg. Ten µl of each extractions were loaded.

Fructan content is high and stable in a transgenic line but not in a high sugar elite variety

The fructan content in a transgenic high-fructan line (N2-6) and a control line (both of N2 genotype) was analysed during a seven month growth period under greenhouse conditions. In the same experiment, the elite variety AberDart from Institute of Grassland and Environmental Research, marketed as a high sugar variety, was included for comparison. All the investigated plants were cloned from one tiller to five plants each allowing one plant to be harvested every month initiated after three months of growth.

Material from the aerial part of the plant above 6 cm from the ground level was harvested on the first day of each following months and the content of WSC was measured. The fructan content increased in all three lines from the third to the fourth months, but the fructan content in AberDart decreased rapidly and remained low for the rest of the test period (figure 3.4).

In contrast, the fructan content remained high in the transgenic line throughout the growth period. The fructan content in the control line increased slowly during growth period, but never reached the high fructan levels seen in the transgenic line. No significant differences in glucose, fructose and sucrose contents were measured in the three different lines (data not shown).

Investigation of factors, which can influence on the fructan content

As illustrated in figure 3.2, different fructan contents in the transgenic lines were measured and in order to investigate possible reasons for this pattern, the copy number of transgene was determined. Similar was the measurable transcription levels of both heterologous as well as homologous genes measured.

Six transgenic lines with fructan levels ranging from low to high were investigated with the help of DNA gel blot analysis analysis to determine a possible influence caused by differences in the copy numbers of the integrated transgene (data not shown). Transgene copy numbers from four to more than nine of the transformed onion genes could be registered, but no correlation between fructan content and the number of integrated fructosyltransferase genes was obvious.

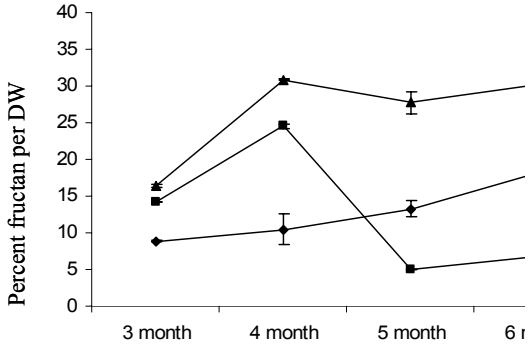

Figure 3.4 Fructan content in a transgenic line, control line and high sugar elite variety.
The amount of fructan in a control line (diamonds), elite variety (AberDart) (squares) and transgenic line (N2-6) with N2 genotype expressing both *Ac1-SST* and *Ac6G-FFT* (triangles) was measured through a growth period. All lines were harvested 6 cm above the ground. Data from transgenic lines were analysed for significant differences from control lines using *t* test. Values are the mean of duplicates extractions ± SE. Where no error bar is included, the error was smaller than the symbol.

The level of measurable transcription of the introduced fructosyltransferase genes, *Ac1-SST* and *Ac6G-FFT* was investigated by Q-PCR and the results of four transgenic lines representing the different levels of fructan contents found in the transgenic lines was correlated to the measurable transcription level of *LpGAPDH* as endogenous control (figure 3.5). The fructan content (white columns) showed no direct correlation to the measurable transcription levels of either *Ac1-SST* (light grey columns) or *Ac6G-FFT* (dark grey columns). For instance, high measurable transcription levels of both genes were measured in transgenic lines with high (N2-6) as well as with low (F6-1) fructan content. Q-PCR analysis of all transgenic lines analysed showed the same lack of correlation between fructan content and levels of transgene measurable transcription (data not shown).

Nevertheless, irrespective of the actual level of transgene measurable transcription, the transgene measurable transcription is prerequisite for high fructan levels since high fructan levels only could be found in transgenic lines with measurable transgene expression, and never in lines without measurable transcription of either of the introduced fructosyltransferases or in control lines (figure 3.2).

Besides the detectable transcription levels of the introduced fructosyltransferases, *Lolium*'s own fructosyltransferases can also participate in the synthesis of fructans in the transgenic lines. Therefore, the detectable transcription levels of two putative fructosyltransferases (*LpFTa* and *LpFTb*) in transgenic and control lines were measured using Q-PCR analysis (figure 3.6).

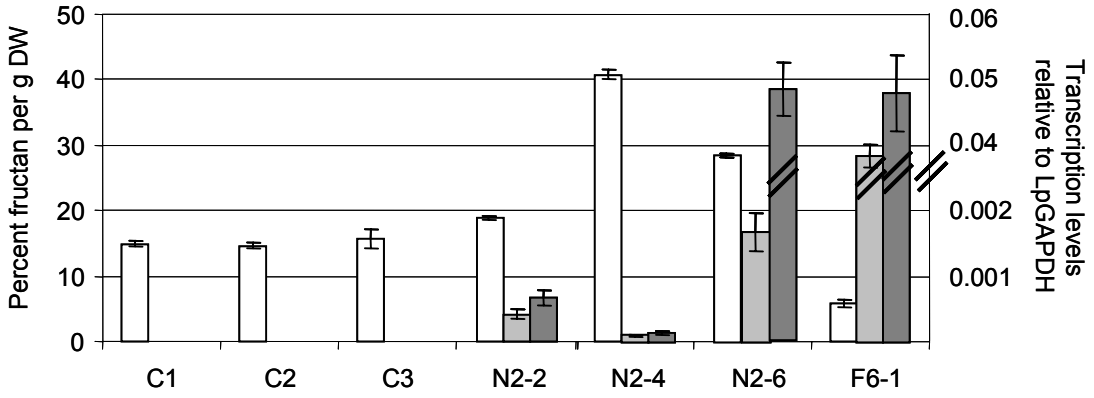

Figure 3.5 Fructan content and transcription level of *Ac1-SST* and *Ac6G-FFT*.
cDNA samples of C1 (N2 genotype), C2 (F6 genotype), C3 (transformed with a control plasmid in F6 genotype) and transgenic lines N2-2, N4, N2-6 and F1 were used to determine transcription level of *Ac1-SST* (light grey boxes) or Ac*6G-FFT* (dark grey boxes) correlated to LpGAPDH and compared with fructan content (white boxes). Data are calculated with the Q-gene software tool (Muller et al. 2002). Values are the mean of triplicates ± SE.

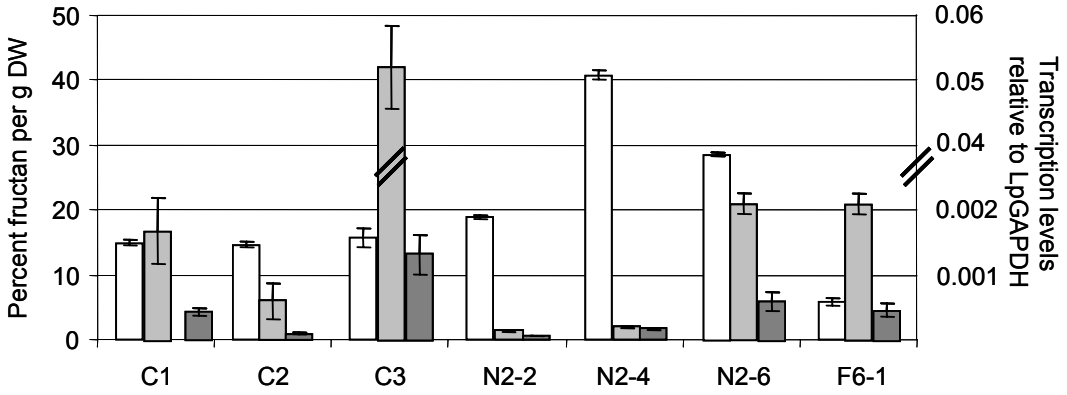

Figure 3.6 Fructan content and transcription level of *LpFTa* and *LpFTb*.
cDNA samples of C1 (N2 genotype), C2 (F6 genotype), C3 (transformed with a control plasmid in F6 genotype) and transgenic lines N2-2, N4, N2-6 and F1 were used to determine transcription level of *Ac1-SST* (light grey boxes) or Ac*6G-FFT* (dark grey boxes) correlated to LpGAPDH and compared with fructan content (white boxes). Data are calculated with the Q-gene software tool (Muller et al. 2002). Values are the mean of triplicates ± SE. Where no error bar is included, the error was smaller than the symbol.

However, in accordance with the results presented for the detectable transcriptional levels of the heterologous fructosyltransferases, no correlation between the detectable transcription level of homologous fructosyltransferases and fructan content could be established. In addition, large differences in detectable transcription levels of *LpFTa* and *LpFTb* were measured in different control lines with similar levels of fructan accumulation. Furthermore, similar detectable transcription levels of the homologous fructosyltransferases were measured in two transgenic lines with different amount of fructan (28 and 6 % per g DW) as illustrated in figure 3.6.

Discussion

Transgenic lines with 3-fold increased fructan content compared to control lines
Since the first plant fructosyltransferase (6-SFT) was isolated from barley in 1995, a number of genes encoding fructosyltransferases have been cloned and used in transgenic studies (reviewed in Cairns, 2003) but only one study has been performed in *L. perenne* using fructosyltransferases genes from wheat (Hisano et al. 2004a). Here, we used two onion genes encoding fructosyltransferases, introduced these into *L. perenne* by co-bombardment and obtained up to 41% fructan content per g DW; a level, which has not previously been previously reported using a transgenic approach in *L. perenne*.

In order to minimise possible variation caused by the experimental design, the control and transgenic lines were cloned from one tiller and after an initial three month growth period in the greenhouse, the plants were harvested at noon on the first day of each successive month. Since the fructan content varies in different plants tissue (Pavis et al. 2001a), it is very important that the same type of plant material is analysed. Therefore, we collected plant material above 6 cm from the ground level in each harvest. Under our growth conditions, the maximum fructan content was measured after five month of growth in three independent experiments (data not shown) and this growth period was used in all further experiments with transgenic lines. Similar number of control and transgenic lines were analysed for fructan content after five months of growth under our greenhouse conditions. In the 23 control lines, consisting of transgenic lines without transgene detectable transcription, untransformed elite varieties and untransformed plants regenerated from callus, the fructan content never reached a level above 15 % per g DW, whereas in 10 out of the 21 transgenic lines with transgene detectable transcription, a significantly higher fructan content compared with the control lines could be measured. This strongly indicates that the increased fructan content is a result of the introduced genes. If the increased fructan content was a result of transformation event, a corresponding number of lines with increased fructan content should have been detected among the group of control lines without transgene detectable transcription (T1-T12). We have no explanation why not all of the transgenic lines with detectable transcription of the introduced genes showed increased fructan content, but this might be attributed to uncontrollable effects caused by the bombardment mediated transformation of the genes into the genome of Lolium. However, the

fact that none of the analysed control lines ever reach fructan content above 15 % per g DW, supports our conclusion that the detectable transcription of the onion fructosyltransferases genes caused the fructan increase in the transgenic lines.

Several of the transgenic lines, and in particular those of N2 genotype, showed significantly higher fructan content compared to the control lines. One explanation of the higher fructan content obtained in transgenic lines of N2 genotype compared with F6 genotype, could be the physiology of the two different genotypes. The N2 genotype is characterised by longer sheath bases and leaves compared to the F6 genotype which could make the N2 genotype more suitable for synthesis and storage of fructans.

The transgenic line with highest fructan content (3-fold more fructan than any of the control lines) showed no visual morphological changes in either genotype used in contrast to plants transformed with bacterial fructosyltransferase (Caimi et al. 1996; Ye et al. 2001). Cairns (2003) suggested that the lack of phenotypic effects observed in plants transformed with plant fructosyltransferase(s) may reflect the low concentration of fructan content achieved in the transgenic lines. However, our results show for the first time high levels of fructans in perennial ryegrass without causing any visual morphologically abnormal or unwanted phenotypes such as growth reduction. Furthermore, the content of glucose, fructose and sucrose was not affected in high fructan transgenic lines compared to the control lines indicating that the synthesis of fructan did not cause any major changes in the synthesis or storage capability of these carbohydrates.

Crude extracts of the transgenic lines with increased fructan content were analysed by thin layer chromatography, and all the transgenic lines with significantly increased fructan content were characterised by a different fructan finger print with fructan DP values which were not present in control lines. However, *L. perenne* contain fructans with high DP values which are very difficult to separate on a TLC plate in the chosen experimental setup (Ye et al. 2001). Therefore, both in the control and the transgenic lines fructans with a high degree of polymerisation did not moved from the loading points into the TLC plate matrix and could not be separated. Nevertheless, the different fructan patterns seen in the high fructan lines indicate the activity of the introduced onion fructosyltransferase genes.

Fructan content is high and stable in a transgenic line compared to a high sugar elite variety

Conventional grass varieties have been developed and marketed as high sugar since 1991 with an average WSC content of 24 % (Wilkins and Lovatt 2004). However, field experiments showed that the high WSC contents in different varieties are not stable during the growth season (Wilkins and Lovatt 2004; G. Gadegaard unpublished results). In our study, an average of the WSC content in seven elite varieties showed 23 % lower fructan content under greenhouse conditions compared to field conditions (G. Gadegaard unpublished results). In contrast to the high sugar elite variety (AberDart), the fructan content in the transgenic N2-6 remained stable high through out the tested growth period. This observation is in agreement with a recent study where the same onion fructosyltransferases were transformed into sugar beet, resulting in a stable fructan content (Weyens et al. 2004).

Variable fructan content in different transgenic lines

It is well known that introducing genes encoding plant fructosyltransferases into plants results in very different levels of accumulated fructans though explanations for this pattern are rarely proposed (Hellwege et al. 2000; Hisano et al. 2004a; Vijn et al. 1997; Weyens et al. 2004). Hisano et al. (2004) discussed possible silencing effects caused by high sequence homology of the introduced fructosyltransferases and the use of the same promoter for two fructosyltransferases genes to be responsible for the low transcription levels and fructan content measured in their study. In this study, we investigated different possible reasons to explain the variation in fructan content obtained in our study.

Determination of transgene copy numbers was performed in six transgenic lines with different fructan levels ranging from low to high. No clear correlation between the fructan content and the number of integrated fructosyltransferase copies could be identified from the DNA gel blot analysis (data not shown). This observation is in agreement with another transgenic study in sugar beet (Weyens et al. 2004).

Another possibility could be different measurable transcription levels of the introduced fructosyltransferases. However, no linear correlation could be measured between the fructan content and transgene measurable transcription levels. Since *L. perenne* is a fructan-accumulating plant, the fructan content obtained in the transgenic

lines could also be influenced by the measurable transcription of the homologous fructosyltransferases. Therefore, two putative fructosyltransferases were cloned (*LpFTa* and *LpFTb*, accession number DQ408726 and DQ408727, respectively) and the measurable transcription levels were analysed in the transgenic lines. However, as for the heterologous fructosyltransferases, no linear correlation could be found between fructan content and measurable transcription levels of the investigated homologous fructosyltransferases. In three control lines with 15 % fructan per g DW, the measurable transcriptional levels of *LpFTa* and *LpFTb* showed large differences despite that sample material for Q-PCR was harvested simultaneously and at the same time as the material for fructan analysis. This indicates that the measurable transcriptional levels of at least *LpFTa* and *LpFTb* cannot be directly correlated to the fructan content.

The lack of a correlation between fructan content and measurable transcriptional levels of either the heterologous or the homologous fructosyltransferases could be explained by the following hypotheses: I) the presence of another fructosyltransferase/fructan exohydrolase gene(s) in *L. perenne* that plays a major role in fructan synthesis, II) post-transcriptional processing of the fructosyltransferase transcripts or III) regulation of fructan synthesis takes place on the enzyme activity level rather than on the measurable transcriptional level.

In conclusion, we have successfully improved the fructan content in perennial ryegrass up to 3-fold, with a maximum level of 41 % per g DW in the plant lines transformed with the onion fructosyltransferase genes *1-SST* and *6G-FFT*. Furthermore, we could demonstrate that the fructan content remained high and stable in the transgenic line compared to an elite variety. Besides the improved fructan content, the transgenic lines contain fructans with additional DP values that could be observed in the control lines demonstrating the activity of the heterologous fructosyltransferases in *L. perenne*.

Acknowledgement

We are grateful to Prof. S. Smeekens for the *Ac1-SST* and *Ac6G-FFT* plasmids and Dr. A. Heyer for the *Cs1-SST* plasmid. We also wish to thank Dr. I.S Oyarzábal for introducing TLC analysis and Prof. D.B. Collinge for critical reading of the manuscript. This work was supported by Danish Functional Genomics Research Initiative for Improving Feed Quality and Disease Resistance in Grasses and Cereals, Ministry of Food, Agriculture and Fisheries (93S-9943-F07-00047).

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3.2 Seasonal changes in water-soluble carbohydrate content in perennial ryegrass varieties through two growth seasons

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Prepared for submission to Grass and Forage Science.

Abstract

Seven perennial ryegrass (*Lolium perenne* L.) varieties were analysed for water-soluble carbohydrate (WSC) content is defined as the sum of fructan, sucrose, fructose and glucose and was measured throughout growth season in 2004 and 2005. The varieties displayed similar patterns of carbohydrate content independent of the production and harvesting year of the different plots. They exhibited high WSC and fructan content during the summer, decreased in mid-summer followed by an increase again in early-autumn. For the rest of the growth period, the WSC stayed more or less constant, whereas the fructan content decreased. The constant WSC content is a result of an increasing amount of fructose following the degradation of fructans. Calculating the percentage fructan as a part of the total amount of WSC, it was observed that the relative content of fructans was in general higher in 2005 than in 2004 independently of the production year the plots. This could be correlated with low amount of rainfall in 2005, especially in August where the relative content of fructans were accumulated most. The different varieties exhibited different fructan levels reflected by the production and harvesting year. However, one variety (Indiana) did contain low fructan content independently of the production and harvesting year, which makes the variety particular suitable for grass mixtures where a low fructan content is preferred. In contrast, varieties as Cancan and Falstaff exhibited the highest average fructan content throughout the growth season in 2004 and 2005. An investigation of the transcription levels of the cloned six putative genes encoding FEHs from this PhD project could be a relevant investigation even though a correlation of transcription levels of these genes and to the fructan content is only speculative.

Keywords: water-soluble carbohydrate, fructans, seasonal changes, *Lolium perenne*.

Abbreviations: *1-FFT*, fructan:fructan 1-fructosyltransferase *1-SST*, sucrose:sucrose 1-fructosyltransferase; *6G-FFT*, fructan:fructan 6G-fructosyltransferase *6-SFT*, sucrose:fructan 6-fructosyltransferase; *FEH*, fructan exohydrolase; DW, dry weight; WSC, water-soluble carbohydrate.

Introduction

Perennial ryegrass (*Lolium perenne* L.) is used as forage grasses for a number of different reasons; digestibility, palatability and resistance to trampling and animal grazing (Wilkins and Humphreys, 2003; Delagarde *et al.*, 2000; Wilman *et al.*, 1996). However, compared to other grasses as *Poa* and *Festuca*, the nutritional value of *L. perenne* is high but still an inefficient utilisation of the grass biomass occurs in the rumen by micro-organisms. This is mainly a result of inadequate energy supplied to the micro-organisms from the carbohydrates (Moorby *et al.*, 2006; Biggs and Hancock, 2001). Since carbohydrates are easily fermented in the rumen, it has been demonstrated that, by increasing the carbohydrate content in grasses, higher milk production in dairy cows and reduced ammonia release to the environment were achieved (Lee *et al.*, 2003; Miller *et al.*, 2001). It is therefore of high interest to increase the carbohydrate content (*e.g.* the fructans) in grasses for use in intensive milk and meat production systems in ruminants. On the other hand, to high sugar content in the fodder can cause the disease laminitis. Especially horses are very sensitive to high WSC/fructan content in the grass (Bailey *et al.*, 2004). Despite considerable

research effort over recent years, the factors inducing laminitis are not understood in their entirety. In contrast to horses, cows are not as sensitive to high fructan induced laminitis but also laminitis cases in cows have been reported (Thoefner *et al.*, 2004; Bailey *et al.*, 2004).

Fructan and starch function as carbohydrate storage in higher plants. In grasses, fructans are the major carbohydrate source, whereas starch only contributes as a minor source (Humphreys *et al.*, 2006; Cairns *et al.*, 2002). Whereas starch is composed of glucose polymers, fructans are composed of fructose polymers linked by β(2,1) and/or β(2,6) linkages. Fructan synthesis is initiated using sucrose as the first substrate and a number of fructosyltransferases. By adding a fructose unit to sucrose, the simplest fructan (1-kestose) with β(2,1)-linkage, is synthesised and this fructan is used for synthesis of the five different classes of fructans; inulin series, inulin neoseries, levan series, levan neoseries and mixed levan series. Levan series can, however, also be synthesised from sucrose by adding a fructose unit in a β(2,6)-linkage. The different classes of fructans are synthesised by the action of four enzymes: sucrose:sucrose 1-fructosyltransferase (1-SST), fructan:fructan 1-fructosyltransferase (1-FFT), 6-fructosyltransferase (6-FT) and fructan:fructan 6G-fructosyltransferase (6G-FFT). Only three classes of fructans have been identified in *Lolium perenne*: a) the inulin series with β(2,1)-linked fructose units and a terminal glucose unit; b) inulin neoseries with β(2,1)-linked fructose units and an internal glucose unit and c) levan neoseries with β(2,6)-linked fructose units and an internal glucose unit (Bonnett *et al.*, 1994; Sims *et al.*, 1992).

Fructan polymers are degraded by the action of fructan exohydrolases, and several isoforms of fructan exohydrolase exist in *Lolium* species degrading β(2,1) and/or β(2,6) linkages (Marx *et al.*, 1997a; Marx *et al.*, 1997b; Bonnett and Simpson, 1995). Despite the fact that *Lolium* species predominately contain β(2,6)-linked fructans, fructan exohydrolase with higher activity against β(2,1)-linkages than β(2,6)-linkages have also been purified. To date, only one gene encoding a fructan exohydrolase-like enzyme has been cloned from *L. perenne* (Chalmers *et al.*, 2005), but so far, no functional characterisation has been published.

The presence of genetic variation in WSC content in forage grasses has been known for some years and to date QTLs for WSC have being identified (Humphreys *et al.*, 2006). Despite several studies, the mechanisms underlying high WSC phenotypes remain unclear (Smith *et al.*, 2004). Therefore, a better knowledge about the existing varieties will improve the breeding strategies.

In this study, we investigate the WSC and fructan content of seven perennial ryegrass varieties sown in two different years and harvested six times throughout the growth season from June to November in 2004 and 2005 in order to obtain WSC and fructan data throughout the two different growth seasons in order to obtain characterisation of the varieties in respect to the WSC content.

Material and methods

Site description and varieties

The experiment was conducted at the DLF-TRIFOLIUM breeding station, Bredeløkke, south-east Zealand, Denmark. Seven diploid perennial ryegrass varieties were examined covering the different heading groups from early to late (table 3.2).

Table 3.2 Description of the examined varieties

Variety	Country	Heading group[a]
Cancan	The Netherlands	8-9
Pastour	France	6
Telstar	Denmark	3
Falstaff	Denmark	8
Lasso	Denmark	6-7
Indiana	The Netherlands	3
Cashel	Ireland	3-4

a: heading group is defined as: 1-3:7th-21th of May (early), 4-5: 22th of May-4th of June (intermediate), 6-8: 5th-17th of June (late) and 9: 18th-24th of June (very late) (M. Greve, personal communication).

The varieties were grown in one plot (1.5 x 8 m) per variety and were harvested six times during each growth season in 2004 and 2005 (named harvest year). The varieties cut in growth season 2004 (harvesting year) were sown in June 2002 and June 2003 (seeding year), whereas the varieties harvested in 2005 were sown in June 2003 and 2004. Thus, all the varieties were harvested and analysed in their first and/or second production year (table 3.3).

Table 3.3 Overview of seeding, production and harvesting year 2004 and 2005.

	Sown in 2002	Sown in 2003	Sown in 2004
Harvest of first production year	n.d.	d. (2004)	d. (2005
Harvest of second production year	d (2004)	d. (2005)	n.d.

d.: determined, n.d. not determined

Based on table 3.3, the varieties can be analysed in the different aspects:

I. 2004: Measuring of the varieties from two different production year (2002 and 2003)
II. 2005: Measuring of the varieties from two different production year (2003 and 2004)
III. 2004 and 2005: Measuring of the varieties from the same production year (2003), but grown for two different growth seasons (2004 and 2005).

Temperature and rainfall were recorded by Danish Meteorological Institute at the public weather station with similar weather conditions (*e.g.* close to the sea) (Figure 3.7).

Collection of samples
Each plot was cut mechanically at 6 cm above ground level. In total, four mechanical harvests were performed each month (June, July, August and September in 2004 and June, July, August and October in 2005) during the growth seasons. The harvests of the different production year were performed with one week's interval and started always with plots from the second production year.

Between each harvest, fertiliser was applied containing 21 % nitrogen, 3 % phosphate, 10 % potassium, 3.6 % sulphur and 1.2 % magnesium (Kemira, Denmark). The fifth (October) and sixth (November) harvests were performed by harvesting five small samples randomly distributed over the plot from each variety to obtain one representative sample by cutting the plants at 6 cm above the ground level. Samples from the fifth harvest were collected after the first night with temperatures below 0ºC. The sixth harvest was performed after several nights with temperatures below 0ºC.

Each harvest was initiated at 11 a.m. and samples for WSC content were collected and kept on dry ice before being stored at -80ºC. This method, instead of immediate freezing in liquid nitrogen, was recently demonstrated to give only a minor, non-significantly drop in the fructan content and had the advantage of simplifying the handling of samples (Narra *et al.*, 2005).

Analysis of water-soluble carbohydrate content
The WSC content is defined as the sum of glucose, fructose, sucrose and fructan. Each carbohydrate is measured as percentage per g DW. The WSC measurements were performed using an enzymatic method as previously described (Gadegaard (2007), PhD thesis chapter 3.1).

Results

Temperature, rainfall and days with frost in 2004 and 2005
The monthly average temperature throughout the growth season in 2004 and 2005 was very similar and did only vary more than between 0.5-2.3 ºC (Figure 1). The amount of rainfall, however, differed greatly between the two years. The growth season in 2005, and especially august, was very dry with only 29 mm precipitation *i.e.* 68 mm less than in august 2004. Throughout the growth season in 2005, the amount of rainfall was 30-80 percent lower than in 2004.

Since 2005 was characterised with low levels of precipitation during the summer, the varieties did not have optimal growth conditions. This resulted in low growth rates after first harvest and is therefore only four or five harvests were performed during 2005. The first and second

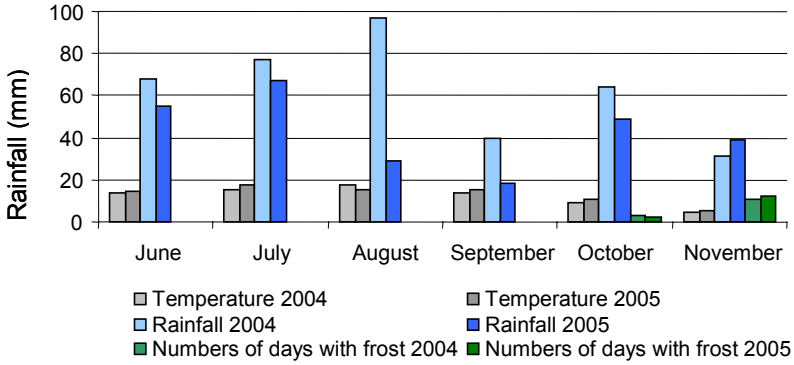

Figure 3.7 Temperature, rainfall and days with frost in 2004 and 2005. Temperature in 2004 (□),temperature in 2005 (■), rainfall in 2004 (□),rainfall in 2005 (■), days with frost in 2004 (■) and days with frost in 2005 (■).
20 mm = 20 ºC = 20 days

harvests were performed at almost the same dates in 2004 and 2005. In 2004, the third harvest was performed at the beginning of August, but not performed before three weeks later in 2005. As a result of the low growth rate, harvest in September 2005 was not performed. Since the third harvest was performed at different dates in 2004 and 2005, the harvests in August are subsequently mentioned as early or late from 2004 or 2005, respectively.

The harvest in October and November was also performed almost at the same dates in 2004 as well as 2005. However, the fifth harvest from the first production year was not cut in 2005, since the field was ploughed in November.

WSC content

The WSC content (the sum of fructan, sucrose, fructose and glucose content) was, for all the varieties cut in 2004, highest in June and July independently on the production year (table 3.3). From the harvest at the beginning of August, a drop in the WSC content could be measured in all the varieties independently of the production year. In September, the majority of the varieties displayed an increase in the WSC content independently of the production year. The WSC content, however, did not reach a higher level than that measured in June or July.

When the autumn starts, the varieties began to exhibit differences depending on the production year. In October, all the varieties, except Indiana, showed a further increase in the WSC content in the plots from the first production year. Opposite, the majority of the varieties, from the plots in second production year, showed a decrease in the WSC content. The WSC content decreased in November in all the varieties, independently of the production year.

In 2005, the WSC content showed a different constitution compared to 2004. In all the varieties, from the plots in the first production year, the highest WSC level was measured in June (table 3.8). From the plots in the second production year, the majority of the varieties contained the highest WSC content in June, except Telstar and Cashel, which contained the highest WSC content in third harvest (late August). The same two varieties showed a different pattern than the other varieties by containing an increased WSC content in July, whereas all the other varieties, independent of the production year, showed a decrease in the WSC content. In the third harvest (late August), the WSC content did not show any consistent development relative to the production year. In October, the WSC content decreased in the majority of the varieties independently of the production year. However, three varieties (Falstaff, Lasso and Cashel), all from the first production year, showed an increased WSC content.

In November, the WSC content rose in the majority of the varieties from the plots in the second production year, except Cashel, which showed a drop in the WSC content.

Fructan content

In 2004, the highest fructan content was measured in June or July depending on the production year (table 3.4). In the first production year, the fructan content was highest in July, whereas it was highest in June in the second production year. Independently on the production year, the fructan content decrease in third harvest (early August) but rose again in September in all the varieties.

In October, the fructan content depended on the year of production. All the varieties, from the plots in the first production year, showed a drop in the fructan content, whereas the fructan content varied in the varieties from plots in the second production year and was increasing in some varieties, decreasing in others or contained a similar fructan content compared to the fructan content in the previously harvest. Independent of the production year, the fructan content decreased in all the varieties in November.

For the majority of the investigated varieties in 2005, the fructan content was highest in June independently on the production year except Telstar, Indiana and Cashel (table 3.9). For these three varieties, the highest fructan content was first measured in third harvest (late August).

For all the varieties from plots in the first production year, the fructan content increased in third harvest (late August). Similarly, the fructan content increased for the majority of the varieties from the plots in the second production year except Falstaff, Lasso and Cashel, which all showed a decrease in fructan content. The opposite pattern was measured in next harvest, since the fructan content decrease in the same varieties where it before was increasing and vice versa. In November, the fructan content in all the varieties from the plots in the second production year was further decreased.

By calculating the percentage of fructan as a part of the total amount of WSC, it could be observed that the percentage share of fructans were very different within the two growth seasons (Figure 3.8 and 3.9).

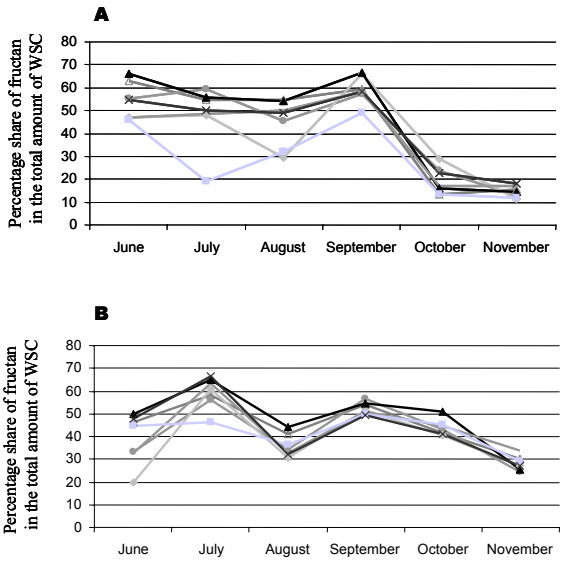

Figure 3.8 Percentage fructan in the total amount of WSC measured in 2004.
A: Varieties from first production year and B: Varieties from second production year.
Cancan (—△—), Pastour (—●—), Telstar (—◆—), Falstaff (—◆—), Lasso (——), Indiana (—■—) and Cashel (—✕—).

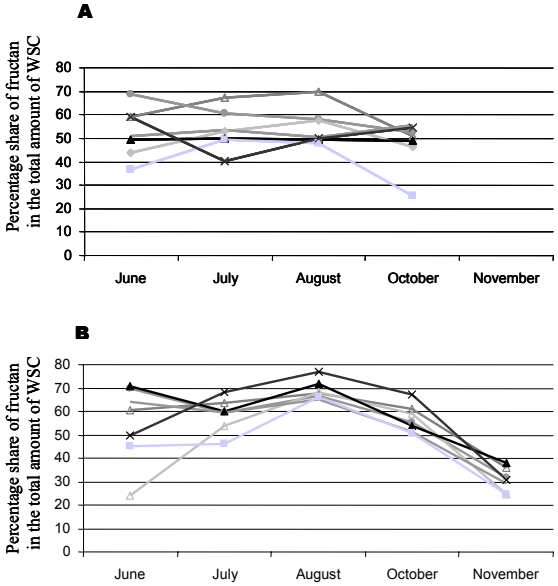

Figure 3.9 Percentage fructan in the total amount of WSC measured in 2005.
A: Varieties from first production year and B: Varieties from second production year.
Cancan (—△—), Pastour (—●—), Telstar (—◆—), Falstaff (—◆—), Lasso (——), Indiana (—■—) and Cashel (—✕—).

Independently of the production and harvesting years, the majority of the varieties showed highest fructan accumulation in late August (2005, figure 3.9) or mid September (2004, figure 3.8) and in general, the accumulation of fructans was higher in 2005 than 2004. One variety (Indiana), however, displayed lowest average fructan content independently of the production or harvesting year.

Sucrose content
The content of sucrose was very different depending on the production and harvest year and was independent of the fructan content (table 3.5 and 3.10). In 2004, the highest sucrose content was measured in June and was generally higher in second production year compared to first production year. In 2005, however, the sucrose content was not higher than eight percent per g DW and the highest content was measured in November.

Fructose content
Independently of the production and harvesting years, the fructose content was highest in the autumn in the majority of the varieties (table 3.6 and 3.11). In 2004, however, all the varieties from second production year did also contain high fructan content in June. This high fructose content was not measured in 2005 independently of the production year.

Glucose content
The glucose content displays the same pattern as sucrose being highest in June (table 3.7) and in November (table 3.12) in harvesting year 2004 or 2005, respectively. As for sucrose, the content of glucose was independently on the production or harvesting years.

Discussion

In many field experiments, the investigated varieties are sown in different areas and cut the same year in order to determine the differences in *e.g.* sugar content as a result of different climate conditions (Smith *et al.*, 2004; Wilman *et al.*, 1996; Humphreys, 1989). Here, we used the same areas, but investigated the varieties through two different years in order to determine the climate influence on the sugar content. However, no replicates within the same production year were performed due to the fixed set up of the different plots, which were available for this field study. Since both growth seasons contained two different production years, we were able to compare the

same varieties, grown under the same conditions, but with two different sward ages. Furthermore, we were able to investigate the varieties sown in the same year but cut in two different growth seasons (see table 3.3 for overview). Also the different weather conditions within the two investigated harvesting year, favourable the characterisation of the varieties under different growth conditions.

Each harvest was initiated at 11.00 PM but since the investigated varieties were placed in the mid or at the end of the field, it could not be avoided that the harvests time point from the varieties differed within the different harvests between two to three hours. However, as the WSC content is a result of photosynthetic activity, it could not be avoid that the WSC content will be higher later at the end of the day compared to at noon. Also the temperature and the amount of sunshine will differ as a result of the climate, so an accurate comparison of the varieties even cut at the same day will not be possible under these circumstances.

From each harvest, the grass was weighted and the yield was collected on a computer. However, since around 50 % of the data from 2004 was unfortunately lost during a computer failure, the yield data could not be used in this study.

For both harvest years, the WSC content was highest in June or July and lower in October and November. This is consistent with other studies of *L. perenne*, where the WSC content in spring was higher than in the autumn (Smith *et al.*, 2004; Wilman *et al.*, 1996; Humphreys, 1989). It was also reported that the spring yields were significantly higher than autumn yields.

The varieties displayed differences according to which month the WSC content was highest, depending on the production year. In the majority of the varieties, the WSC content was lower in plots from the second production year compared to the first production year for both growth seasons. This is in agreement with previous studies of *L. perenne* which reported that the WSC content was decreasing with plant age (Turner *et al.*, 2006; Taweel *et al.*, 2005; Wilman *et al.*, 1996).

Since the content of fructans is the major contributor to the WSC content, the pattern of fructan influences the WSC content. Calculating the percentage of fructan content as part of the total amount of WSC, it could be observed that the percentage share of fructan is highest in late August or mid September in 2005 and 2004, respectively for the majority of the varieties. Only the varieties from the plots in the second production year in 2004 have a higher percentage of fructan content in July than in September. The level of fructan was in general higher in 2005 compared to 2004 and did not show the same fluctuations as in 2004. This could be a result of the differences in the climate in the two years. There was less rain in 2005, than in 2004 and this could coincide with the fact that fructans are expected to accumulate initiated by drought (Amiard *et al.*, 2003; Wilson and Kachman, 2001; De Roover *et al.*, 2000; Thomas and James, 1999).

It has been reported that fructans are degraded in the autumn as a result of decreasing temperatures (Wilson *et al.*, 2004). In this study, a drop in the fructan content could also be measured in the autumn in both years. However, the month in which the decrease in the fructan content was observed depended on the production year. In 2004, the decrease was measured in October for the second production year, whereas the decrease was first measured in November in the plots from the first production year. This pattern was different in 2005; the fructan content decreased in October for all the varieties from the plots in the first production year, whereas not all of the varieties from the plots in the second production year displayed a decrease. This could be a response to the winter hardness of the varieties since the winter hardness is higher in the first production year compared with the second production year (M. Greve, personal communication).

In the same period, an increase in the fructose content could be measured independently of the production and the harvest year. This indicates that the decrease in fructan content might be a result of fructan degradation. However, in the second production year (2004), where the fructan content did not decrease, an increase in the fructose content could be measured too. This could indicate that the fructose content also increased as a result of other factors and not only because of the degradation of fructans. In November, where a fructan drop was measured independently of the production and harvesting year, the fructose content rose in the varieties from both production years.

Based on the heading dates, the WSC and fructan content was lower in the early varieties, whereas the late varieties contained higher fructan content in the first harvest. It could therefore be relevant to compose the grass mixtures with varieties with different heading dates in order to obtain a more consistent WSC/fructan content through a growth season.

None of the other sugars (glucose, fructose and sucrose) investigated in this study showed a response to the amount of rain. Similar results have

been reported from another field study (Wilson and Kachman, 2001).

Independently of the sowing and harvesting year, Indiana is the variety with the lowest WSC and fructan content, when calculating the average of the WSC and fructan content in each variety throughout a growth season. Indiana also contained the lowest percentage of fructan in the total amount of WSC. This makes the variety useful in grass mixtures where a low WSC/fructan content throughout a growth season is preferred. This is especially important in grass mixtures used for grazing of horses, since those animals are sensitive to high WSC (fructan) content in the grass which is supposed to initiate laminitis (Bailey *et al.*, 2004).

If high WSC and/or fructan content are preferred, the selection of varieties is more critical since the WSC and fructan content seems to be more affected by the production year and climate. In 2004, the variety with the highest average WSC content was Pastour, followed by Cancan and Falstaff for the first and second production year, respectively. However, in 2005, Cancan was the variety with the highest average WSC content independent of the year of production. Similarly, Cancan was also the variety with highest fructan content in 2004 and 2005. Cancan and Falstaff contained almost the same fructan level, which could reflect the heading date. However, the yield of Cancan and Falstaff will not be high in the first production year, because of their heading date.

Based on these results, Cancan and Falstaff will be a good choice, when high WSC and/or fructan are preferred for grazing of ruminants through a growth season. In addition, Cancan is also defined to be late in the flowering development, which also increases the nutritional value, since flower stems decrease the nutritional value making the varieties suitable for grazing of ruminants. Besides late production of flower stems, high WSC/fructan content seems to be positive for increasing the milk and meat production in ruminants (Miller *et al.*, 2001). However, also a factor as palatability is important, when selecting varieties for grazing of ruminants.

In conclusion, we have determined the WSC (incl. fructan, sucrose, fructose and sucrose) content in seven different varieties, grown in same areas but from different production and harvest years. The results demonstrate that the climate is affecting the WSC content and that the varieties contained different levels of WSC depending on the climate. Also the production year affects the WSC content, giving the highest WSC level in varieties from the plots in the first production year compared to the second production year. The feature was independent on the growth season. The climate did also affect the amount of fructan accumulated and the results clearly demonstrated that the fructan content was increased during drought.

However, despite of the different amount of the WSC content through the two growth season, Indiana was characterised by having a low average WSC content, whereas Cancan and Falstaff contain high levels of WSC.

As a result of the impact of the climate on the WSC content, it is important to characterise the varieties through several growth seasons. Therefore, the field studies on the seven elite varieties have been continued after this study by DLF-TRIFOLIUM.

Acknowledgement

To Morten Greve for selection of the varieties examined in this study and fruitful discussions and to Christian F. Mogensen for all mechanical field harvests. This work was supported by Danish Functional Genomics Research Initiative for Improving Feed Quality and Disease Resistance in Grasses and Cereals, Ministry of Food, Agriculture and Fisheries (93S-9943-F07-00047).

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Table 3.3 Seasonal variation in WSC content for perennial ryegrass varieties under a conservation management in 2004

Variety		Cancan		Pastour		Telstar		Falstaff		Lasso		Indiana		Cashel	
Harvest number	Harvest date	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*
1	2nd June	44.5±0.12	27.5±0.06	26.3±2.66	28.3±0.13	33.7±6.43	26.0±0.51	48.5±1.94	33.5±0.42	31.3±3.34	30.5±0.20	41.3±0.70	35.8±0.26	35.8±0.92	33.4±0.66
2	7th July	26.5±0.18	34.0±2.04	29.1±0.54	25.2±0.05	26.2±1.03	34.8±0.23	25.5±0.41	30.1±2.01	25.5±0.81	32.1±1.70	18.8±0.57	23.9±1.85	27.7±0.40	35.3±1.89
3	4th August	23.2±0.57	23.3±0.30	17.8±0.13	19.5±1.06	14.4±0.27	15.8±0.14	20.2±1.20	18.5±1.43	23.5±0.22	21.0±0.04	16.2±0.00	18.7±0.27	22.5±0.89	22.2±1.14
4	16th September	27.3±0.94	21.5±0.62	23.6±0.39	30.4±2.43	30.3±1.82	22.8±0.67	29.4±2.14	21.5±1.95	22.2±0.54	25.1±0.49	20.8±0.28	19.1±0.73	22.5±1.83	20.4±1.55
5	20th October	21.7±0.19	27.8±4.00	23.3±0.04	31.7±2.19	21.4±0.11	33.6±1.11	19.0±1.18	23.9±2.86	22.3±0.69	29.9±0.54	21.9±0.54	17.9±1.81	23.1±0.01	22.7±1.06
6	15th November	17.6±1.33	21.2±0.95	17.5±0.33	17.2±0.02	18.8±0.09	19.4±0.39	18.2±0.43	18.2±0.70	17.6±0.26	19.5±0.88	13.5±0.28	17.2±0.31	16.0±0.19	17.6±0.40
	Average	26.8	25.9	22.9	27.1	24.1	25.4	26.8	24.3	23.7	26.4	22.1	22.1	24.6	25.3

* The harvests in the second production year were performed within a week after the harvest of first production year.

Table 3.4 Seasonal variation in fructan content for perennial ryegrass varieties under a conservation management in 2004

Variety		Cancan		Pastour		Telstar		Falstaff		Lasso		Indiana		Cashel	
Harvest number	Harvest date	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*
1	2nd June	28.0±0.04	12.8±0.45	14.6±1.62	9.45±0.06	15.9±5.81	5.38±0.47	32.1±1.35	16.4±0.48	14.7±1.50	10.6±0.07	18.9±0.55	16.4±0.64	19.6±0.37	16.0±0.18
2	7th July	14.5±0.30	20.2±0.06	17.2±0.30	13.9±0.35	12.6±0.64	21.0±0.48	14.2±0.11	19.8±0.95	12.4±0.48	19.0±2.16	3.60±0.17	11.6±0.00	13.9±0.34	23.0±1.92
3	4th August	12.7±0.72	9.44±0.09	8.05±0.06	6.72±0.36	4.25±0.25	5.06±0.34	10.9±0.06	8.14±0.62	11.8±0.04	7.05±0.25	5.18±0.15	6.97±0.34	11.0±0.37	7.44±0.76
4	16th September	16.2±0.56	11.5±0.19	13.6±0.21	17.2±1.48	20.0±2.06	11.8±0.17	19.6±0.32	12.2±1.68	13.0±0.29	12.7±0.19	10.2±0.14	9.31±0.72	13.1±1.45	9.87±1.05
5	20th October	2.85±0.21	11.5±1.68	5.55±0.00	13.4±1.32	6.20±0.06	12.7±0.71	3.05±0.24	12.0±1.21	3.81±0.29	13.3±0.13	2.96±0.01	7.45±1.77	5.30±0.71	9.79±1.09
6	15th November	2.72±0.47	6.25±0.01	2.27±0.12	3.93±0.33	2.15±0.18	5.50±0.52	2.58±0.15	4.53±0.09	2.96±0.05	6.61±0.29	1.58±0.06	4.77±0.37	2.90±0.15	4.76±0.11
	Average	12.8	11.9	10.2	10.8	10.2	10.2	13.7	12.2	9.78	11.5	7.07	9.42	11.0	11.8

* The harvests in the second production year were performed within a week after the harvest of first production year.

Table 3.5 Seasonal variation in sucrose content for perennial ryegrass varieties under a conservation management in 2004

Variety		Cancan		Pastour		Telstar		Falstaff		Lasso		Indiana		Cashel	
Harvest number	Harvest date	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*
1	2nd June	7.91±0.05	1.16±0.00	1.75±0.22	1.56±0.30	7.64±0.83	0.16±0.01	9.24±0.13	2.17±0.15	2.60±0.38	0.99±0.00	0.77±0.31	1.72±0.37	7.88±0.02	2.52±0.57
2	7th July	2.01±0.14	3.83±1.65	1.54±0.19	1.18±0.03	2.28±0.13	3.99±0.41	2.12±0.15	1.21±0.35	1.08±0.01	2.34±0.63	0.00±0.00	2.43±1.14	8.46±0.12	2.90±0.28
3	4th August	1.26±0.04	1.44±0.56	1.08±0.02	1.80±0.12	1.20±0.06	3.52±0.02	0.80±0.16	4.12±0.28	2.02±0.08	2.45±0.18	1.30±0.05	4.51±0.30	1.27±0.11	3.22±0.25
4	16th September	2.49±0.12	1.02±0.01	2.73±0.04	1.99±0.46	3.64±0.11	2.45±0.44	2.55±0.35	1.90±0.21	2.25±0.03	1.84±0.45	2.51±0.23	2.03±0.05	2.47±0.43	2.88±0.54
5	20th October	5.44±0.13	3.56±0.45	5.07±0.56	3.03±0.66	4.85±0.17	2.85±0.48	4.06±0.58	3.01±0.53	4.96±0.46	2.85±0.04	6.75±0.64	2.47±0.24	5.13±0.24	3.78±0.31
6	15th November	2.11±0.20	2.03±0.21	1.82±0.02	1.32±0.25	2.19±0.09	1.86±0.17	1.99±0.43	1.91±0.04	1.36±0.02	1.58±0.30	2.07±0.09	1.33±0.02	2.43±0.12	1.86±0.28
	Average	3.54	2.17	2.33	1.81	3.63	2.47	3.46	2.39	2.38	2.01	2.23	2.42	4.61	2.86

* The harvests in the second production year were performed within a week after the harvest of first production year.

Table 3.6 Seasonal variation in fructose content for perennial ryegrass varieties under a conservation management in 2004

Variety		Cancan		Pastour		Telstar		Falstaff		Lasso		Indiana		Cashel	
Harvest number	Harvest date	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*
1	2nd June	4.85±0.11	8.46±0.07	5.65±0.50	9.20±0.12	5.83±0.15	10.9±0.07	4.17±0.27	8.57±0.04	7.43±0.09	12.2±0.08	12.1±0.06	9.74±0.48	4.56±0.38	7.50±0.03
2	7th July	5.71±0.21	5.63±0.73	5.88±0.29	4.95±0.58	6.41±0.13	5.96±0.06	5.68±0.07	4.46±0.65	7.71±0.28	5.08±0.54	8.87±0.28	5.33±0.91	3.03±0.10	4.96±0.08
3	4th August	6.11±0.10	7.48±0.66	5.37±0.11	6.35±0.37	5.18±0.05	3.93±0.26	4.91±0.72	3.17±0.27	5.67±0.03	6.60±0.04	5.76±0.12	3.84±0.13	6.63±0.44	6.65±0.43
4	16th September	4.90±0.16	5.00±0.25	4.25±0.07	5.98±0.31	3.58±0.08	4.62±0.04	3.91±0.85	3.85±0.00	3.77±0.20	5.66±0.07	4.35±0.22	4.07±0.01	3.68±0.02	4.06±0.03
5	20th October	7.88±0.09	7.63±1.24	7.35±0.03	9.38±0.26	6.12±0.04	11.0±1.02	7.22±0.22	5.21±0.72	7.97±0.08	8.22±0.31	6.90±0.07	4.22±0.48	7.40±0.01	5.23±0.24
6	15th November	7.55±0.38	8.20±0.54	7.87±0.07	7.28±0.44	9.07±0.59	7.33±0.04	8.06±0.12	7.13±0.42	7.67±0.23	6.83±0.16	5.55±0.08	6.46±0.04	6.15±0.00	6.39±0.00
	Average	6.17	7.07	6.06	7.19	6.03	7.29	5.66	5.40	6.70	7.43	7.26	5.61	5.24	5.80

* The harvests in the second production year were performed within a week after the harvest of first production year.

Table 3.7 Seasonal variation in glucose content for perennial ryegrass varieties under a conservation management in 2004

Variety		Cancan		Pastour		Telstar		Falstaff		Lasso		Indiana		Cashel	
Harvest number	Harvest date	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*	2002	2003*
1	2nd June	3.67±0.02	6.26±0.10	4.32±0.32	8.13±0.11	4.35±0.07	9.53±0.02	3.02±0.19	6.29±0.04	6.58±0.57	7.15±0.05	9.50±0.10	7.96±0.28	3.75±0.15	7.41±0.06
2	7th July	4.13±0.13	3.85±0.27	4.44±0.14	5.14±0.21	5.04±0.06	3.84±0.23	3,54±0.07	4.70±0.05	4.33±0.60	4.78±0.71	5.36±0.12	4.55±0.20	2.36±0.70	4.40±0.17
3	4th August	3.17±0.01	4.98±0.30	3.31±0.50	4.61±0.21	3.80±0.40	3.26±0.20	3.57±0.58	3.06±0.26	4.07±0.06	4.95±0.01	3.91±0.08	3.38±0.10	3.54±0.18	4.89±0.20
4	16th September	3.74±0.10	4.00±0.18	3.74±0.06	5.21±0.18	3.07±0.06	3.87±0.02	3.29±0.63	3.53±0.07	3.23±0.80	4.86±0.07	3.76±0.15	3.73±0.03	3.22±0.03	3.63±0.02
5	20th October	5.37±0.02	5.09±0.63	5.33±0.01	5.95±0.05	4.23±0.07	7.02±0.33	4.69±0.13	3.70±0.40	5.52±0.03	5.51±0.06	5.28±0.03	3.75±0.28	5.27±0.01	3.88±0.10
6	15th November	5.26±0.29	4.75±0.18	5.56±0.13	4.68±0.16	5.36±0.40	4.71±0.02	5.54±0.03	4.59±0.23	5.58±0.07	4.52±0.13	4.27±0.05	4.60±0.04	4.47±0.08	4.61±0.01
	Average	4.22	4.82	4.45	5.62	4.31	5.37	3.94	4.31	4.89	4.30	5.35	4.66	3.77	4.80

* The harvests in the second production year were performed within a week after the harvest of first production year.

Table 3.8 Seasonal variation in WSC content for perennial ryegrass varieties under a conservation management in 2005

Variety		Cancan		Pastour		Telstar		Falstaff		Lasso		Indiana		Cashel	
Harvest number	Harvest date	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*
1	1th June	36.5±0.46	37.8±0.68	40.6±2.24	41.1±0.16	31.3±0.62	23.2±0.11	30.8±0.37	40.9±1.92	30.4±0.37	39.4±0.54	29.0±0.58	28.7±0.53	34.7±0.97	25.1±0.27
2	11th July	27.7±0.35	31.4±0.52	17.6±0.42	26.5±0.13	23.9±0.08	26.2±0.56	21.5±0.59	23.6±0.37	21.2±0.44	29.6±0.70	13.8±0.27	22.1±0.61	25.1±0.36	29.8±0.30
3	29th August	34.6±0.00	30.8±0.02	25.4±0.55	26.0±0.62	22.2±0.37	30.4±1.75	16.2±0.66	24.6±1.59	20.8±0.07	27.3±0.35	16.5±0.02	26.5±0.82	19.6±0.02	36.8±0.59
4	14th October	29.7±0.75	29.4±0.85	22.5±2.92	24.7±0.28	19.1±0.35	24.3±0.25	22.3±0.03	20.6±0.10	27.1±0.48	21.2±0.79	15.2±0.42	20.3±0.08	27.5±0.12	32.8±0.08
5	18th November	n.d.	30.8±0.16	n.d.	29.5±0.37	n.d.	27.6±0.49	n.d.	33.0±1.36	n.d.	30.4±0.77	n.d.	25.5±0.48	n.d.	26.8±0.93
	Average	32.1	32.0	26.5	29.6	24.1	26.3	22.7	28.5	24.9	29.6	18.6	24.6	26.7	30.3

* The harvests in the second production year were performed within a week after the harvest of first production year.

Table 3.9 Sasonal variation in fructan content for perennial ryegrass varieties under a conservation management in 2005

Variety		Cancan		Pastour		Telstar		Falstaff		Lasso		Indiana		Cashel	
Harvest number	Harvest date	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*
1	1th June	21.6±0.62	22.8±0.32	29.7±2.41	28.6±0.40	13.8±0.39	5.62±0.20	15.2±0.03	28.9±0.49	15.5±0.24	25.3±0.03	10.6±0.06	13.0±0.34	20.5±1.27	12.5±0.08
2	11th July	18.6±0.17	20.0±0.31	10.6±0.21	15.8±0.08	12.7±0.04	14.1±0.48	10.7±0.44	14.1±0.12	11.4±0.29	17.6±0.52	6.81±0.14	10.2±0.49	10.1±0.40	20.2±0.05
3	29th August	24.2±0.13	20.8±0.26	14.8±0.32	17.3±0.27	12.7±0.20	20.8±1.32	8.01±0.26	17.7±0.47	10.5±0.11	17.9±0.31	7.89±0.12	17.6±0.59	9.71±0.02	28.4±0.37
4	14th October	15.2±0.55	17.9±0.53	11.8±1.78	13.7±0.12	8.87±0.13	14.3±0.18	10.9±0.20	11.1±0.24	15.0±0.28	10.8±0.52	3.87±0.24	10.3±0.06	15.0±0.23	21.9±0.10
5	18th November	n.d.	11.0±0.05	n.d.	9.43±0.15	n.d.	6.79±0.47	n.d.	12.6±0.69	n.d.	8.83±0.65	n.d.	6.28±0.35	n.d.	8.20±0.32
	Average	19.9	18.5	16.7	17.0	12.0	12.3	11.2	16.9	13.1	16.1	7.29	11.5	13.8	18.2

* The harvests in the second production year were performed within a week after the harvest of first production year.

Table 3.10 Seasonal variation in sucrose content for perennial ryegrass varieties under a conservation management in 2005

Variety		Cancan		Pastour		Telstar		Falstaff		Lasso		Indiana		Cashel	
Harvest number	Harvest date	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*
1	1th June	3.79±0.97	3.59±0.34	3.89±0.07	3.78±0.46	3.39±0.25	3.06±0.22	3.14±0.41	4.09±1.48	3.63±0.51	4.97±0.58	4.10±0.29	4.34±0.85	3.32±0.26	4.30±0.38
2	11th July	2.07±0.11	3.13±0.17	0.82±0.13	3.30±0.11	2.42±0.05	2.82±0.20	2.80±0.07	1.79±0.27	1.99±0.03	3.78±0.16	1.53±0.03	3.00±0.40	0.35±0.10	3.14±0.27
3	29th August	2.58±0.00	2.39±0.03	1.99±0.36	1.47±0.29	1.26±0.15	1.57±0.52	0.95±0.31	1.11±0.79	1.21±0.00	1.97±0.01	0.70±0.11	1.59±0.32	1.93±0.00	2.56±0.31
4	14th October	2.55±0.32	2.72±0.05	1.17±0.72	2.62±0.00	1.47±0.05	1.71±0.02	1.34±0.22	1.25±0.03	2.36±0.22	1.71±0.33	0.77±0.03	2.19±0.14	3.61±0.26	4.05±0.03
5	18th November	n.d.	5.78±0.13	n.d.	5.84±0.33	n.d.	5.41±0.09	n.d.	6.08±0.70	n.d.	5.13±0.02	n.d.	6.56±0.16	n.d.	7.82±0.60
	Average	2.75	3.52	1.97	3.40	2.14	2.91	2.06	2.86	2.30	3.51	1.78	3.54	2.30	4.37

* The harvests in the second production year were performed within a week after the harvest of first production year.

Table 3.11 Seasonal variation in fructose content for perennial ryegrass varieties under a conservation management in 2005

Variety		Cancan		Pastour		Telstar		Falstaff		Lasso		Indiana		Cashel	
Harvest number	Harvest date	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*
1	1th June	6.57±0.06	6.61±0.02	5.07±0.17	5.11±0.08	7.59±0.04	8.38±0.22	7.33±0.08	4.67±0.02	6.53±0.05	5.02±0.01	8.46±0.18	6.57±0.06	6.44±0.00	4.49±0.02
2	11th July	3.55±0.03	4.68±0.01	3.23±0.03	4.27±0.06	4.67±0.00	5.52±0.08	3.95±0.06	4.56±0.02	4.08±0.08	4.64±0.01	2.70±0.08	5.05±0.19	4.87±0.09	3.56±0.07
3	29th August	4.42±0.10	4.37±0.22	4.79±0.02	4.08±0.04	4.54±0.01	4.60±0.06	4.26±0.00	3.22±0.16	4.93±0.05	4.26±0.04	4.41±0.07	4.12±0.07	4.22±0.01	3.18±0.09
4	14th October	7.21±0.07	4.99±0.18	5.78±0.04	4.56±0.12	4.84±0.13	4.52±0.01	5.78±0.03	4.61±0.11	5.41±0.02	4.87±0.04	6.16±0.09	4.19±0.02	4.81±0.10	3.40±0.00
5	18th November	n.d.	7.59±0.02	n.d.	8.23±0.13	n.d.	9.08±0.08	n.d.	7.84±0.00	n.d.	9.00±0.07	n.d.	7.04±0.06	n.d.	5.88±0.00
	Average	5.44	5.65	5.22	5.25	5.41	6.42	5.33	4.98	5.24	5.56	5.43	5.39	5.09	4.10

* The harvests in the second production year were performed within a week after the harvest of first production year.

Table 3.12 Seasonal variation in glucose content for perennial ryegrass varieties under a conservation management in 2005

Variety		**Cancan**		**Pastour**		**Telstar**		**Falstaff**		**Lasso**		**Indiana**		**Cashel**	
Harvest number	Harvest date	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*	2003	2004*
1	1th June	4.50±0.05	4.74±0.04	3.78±0.07	3.61±.02	6.54±0.01	6.16±0.10	5.07±0.04	3.25±0.03	4.69±0.05	4.14±0.02	5.77±0.17	4.78±0.04	4.41±0.04	3.83±0.01
2	11th July	3.44±0.04	3.65±0.06	2.90±0.05	3.16±0.00	4.11±0.01	3.76±0.20	4.01±0.02	3.13±0.00	3.76±0.04	3.52±0.00	2.75±0.07	3.86±0.10	4.80±0.03	2.82±0.08
3	29th August	3.38±0.04	3.23±0.09	3.89±0.10	3.17±0.02	3.77±0.02	3.45±0.03	3.58±0.01	2.64±0.17	4.12±0.01	3.24±0.00	3.51±0.03	3.27±0.02	3.68±0.04	2.75±0.00
4	14th October	4.73±0.05	3.70±0.09	4.19±0.01	3.82±0.03	3.96±0.05	3.78±0.07	4.19±0.02	3.65±0.05	4.30±0.01	3.77±0.02	4.44±0.07	3.55±0.01	4.09±0.05	3.37±0.01
5	18th November	n.d.	6.44±0.04	n.d.	6.04±0.06	n.d.	6.30±0.19	n.d.	6.53±0.03	n.d.	7.48±0.06	n.d.	5.59±0.03	n.d.	4.93±0.02
	Average	4.01	4.35	3.69	3.96	4.60	4.69	4.21	3.84	4.22	4.43	4.12	4.21	4.25	3.54

* The harvests in the second production year were performed within a week after the harvest of first production year.

3.3 Forfangenhed og sukker i græsset (Danish, English summary below)

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Publiceret i Tölt, maj 2005, side 58-60.

Mange heste og ponyer og især de islandske heste rammes hver sommer af forfangenhed. I 2004 var problemet særligt stort pga. regnfulde perioder og gode vækstbetingelser for græsset. Men hvad er det i græsset, som hestene ikke kan tåle? Sidste efterår mistede den ene af forfatterne til denne artikel sin 18-årige islandske vallak pga. forfangenhed, hvilket gav anledning til en nærmere undersøgelse af problemet.

Forfangenhed er en kronisk sygdom – forebyggelse er nødvendig

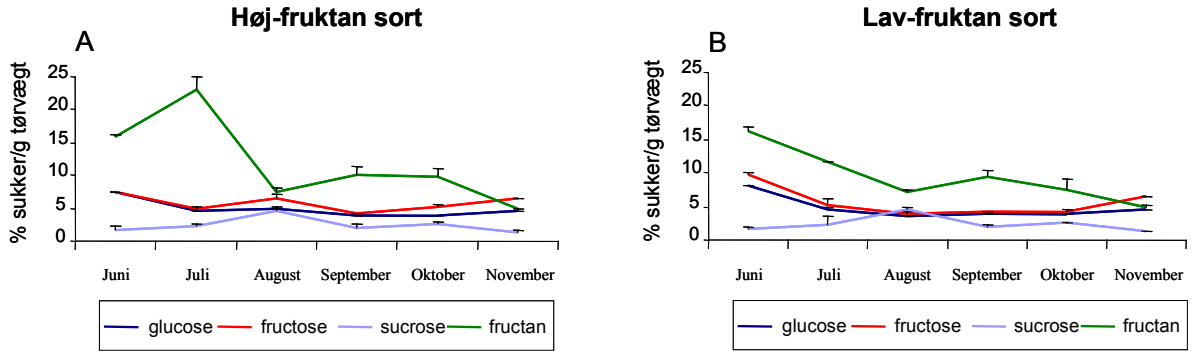
Forfangenhed er næst efter kolik den hyppigste dødsårsag hos heste. Forfangenhed er en kritisk sygdom, idet den ofte er kronisk og svær at kontrollere. Det er typisk heste med tendens til fedme og manglende appetit kontrol, der bliver forfangne. Forfangenhed er desuden blevet mere udbredt de senere år og rammer ikke kun om foråret men også i efteråret. Forklaring kan være svær at finde, men ny forskning skaber bedre forståelse og mulighed for at afhjælpe problemet.

Proteinindholdet i græsset er ofte fejlagtigt antaget som grunden til forfangenhed (1), men den nyeste viden viser, at der en direkte sammenhæng med det vandopløselige sukkerindhold i græsset og forfangenhed (1,2). I Australien har undersøgelser vist, at når forfangenhed indtræder om foråret er særligt sukkerstoffet fruktan en meget vigtig årsag (2). Problemet er, at fruktan ikke fordøjes i tyndtarmen, men derimod gæres mikrobielt i stortarmen, hvilket forårsager en ændring af bakteriebestanden og en sænkning af pH. På den måde vil fruktan-nedbrydende bakterier udkonkurrere andre bakterier og producere toksiner. Disse føres, hos en disponeret hest, via tarmvæggen til blodbanerne og ender i hoven. Her forårsager toksinerne produktion af en større mængde enzymer, som nedbryder hovvæggen (2). Resultatet er, at hesten først bliver halt, derpå ude af stand til at røre sig pga. smerter og i værste fald får varige mén i form af nedsunket hovben eller må slås ned. Det kritiske punkt er, at når skaden først er sket, er den uoprettelig (2). Således er det yderst vigtigt at forebygge med forfangenhed, idet en egentlig helbredelse er tvivlsom.

Indholdet af fruktan i græsset

De fleste kommercielle græsser indeholder store mængder fruktan. En af årsagerne er at landmændene (forståeligt nok) efterlyser græsser med højt fruktanindhold til malkekøer, da et højt fruktanindhold giver en større mælkeproduktion samtidigt med at koen udskiller en lavere mængde ammoniak til miljøet. Desuden er fruktanholdige græsser mere modstandsdygtige overfor tørke og kulde. Det betyder, at de græsblandinger der anvendes i dag med stor sandsynlighed vil være forædlet med henblik på et højt sukkerindhold.

Ser man på sukkerindholdet i græssorter, dyrket i Danmark på DLF-TRIFOLIUMs forsøgsmarker, varierer det med årstiden. Indholdet af vandopløselige sukre afhænger af sorten, hvilket ses illustreret i Figur 1. En høj-fruktan sort (Figur 3.10.A) har det højeste fruktanindhold i 2. slæt der blev høstet i juli 2004, mens en lav-fruktan sort (Figur 3.10.B) har det højeste fruktan indhold målt i 1. slæt, der blev høstet i juni 2004. Begge sorter er karakteriseret ved at fruktanindholdet er lavt i 3. slæt (august) og samtidigt ses der en stigning i sukrose-indholdet. Da fruktan dannes ud fra sukrose, er denne stigning et udtryk for, at der ikke dannes samme mængder fruktan i august måned sammenlignet med de andre måneder. Ligeledes er



Figur 3.10 Indholdet af vandopløselige sukre in sæsonen juni-november 2004 i to forskellige sorter fra DLF-TRIFOLIUM.

begge sorter karakteriseret ved, at indholdet af fruktan stiger hen mod efteråret (september) for så at aftage igen. Det lave fruktanindhold i 6. slæt skyldes nætter med frost. Lave temperaturer aktiverer et planteenzym, der nedbryder fruktan til fruktose, og der kan også observeres en stigning i fruktoseindholdet i begge sorter.

Disse observationer er baseret på rene sorter. Græsblandinger indeholder altid en blanding af forskellige sorter, men undersøgelser af i alt 8 forskellige sorter fra DLF-TRIFOLIUM har vist samme tendens til en stigning af fruktanindholdet i efteråret. Sukkerindholdet i græs afhænger meget af dyrknings forhold og klima (3). Det betyder, at de samme sorter/græsblandinger kan have varierende sukkerindhold fra år til år.

Det er vigtigt at have disse observationer i betragtning, når man vurdere antallet af timer, hestene kan gå på fold i såvel foråret som efteråret. Ved periodevis anvendelse af jordfold til forfangne eller for fede heste er hø eller wrap af god kvalitet (godt bjerget) fortsat et nødvendigt grovfoder og skal gives i mængder svarende til ca. 1 kg pr 100 kg kropsvægt pr. dag (4). Dog kan en brat overgang til wrap fremprovokere forfangenhed igen (5). Desuden anbefales tilskud af vitaminer og mineraler til alle heste både i vinter- og sommerhalvåret, da græs, hø og wrap ikke altid indeholder tilstrækkeligt med vitaminer og mineraler (4,6). Især vitamin A, C og E anbefales som ekstra tilskud til forfangne heste (5). Halm, som ikke kan erstatte hø og wrap pga. meget mindre mængder af næringsstoffer, er et værdifuldt supplement som beskæftigelse for fordøjelsen idet en hest helst skal tygge 15-16 timer i døgnet af hensyn til spyt- og enzymdannelse (4).

På DLF-TRIFOLIUMs hjemmeside (7) kan man læse mere om græsblandinger anbefalet til hestehold. Disse blandinger er sammensat i samarbejde med Dansk Landbrugsrådgivning i Skejby, hvor både afdelingen for grovfoder og afdelingen for heste har været involveret.

Som det også fremgår af DLF-TRIFOLIUMs hjemmeside, er der flere faktorer end sukkerindhold der tages i betragtning, når man sammensætter en god græsblanding til hestehold, f.eks. det samlede udbytte, tolerancen overfor (tæt) nedbidning og varighed. Som afslutning på det 3 årige phd-projekt, der udarbejdes fra 2003 til 2006 i samarbejde mellem DLF-TRIFOLIUM og Afdelingen for Biosystemer på Forskningscenter Risø, forventer DLF-TRIFOLIUM at kunne annoncere en lav-fruktan blanding.

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Laminitis, an inflammation of the sensitive laminae in the hoof, is a disease which affects horses and especially ponies in the spring, when the animals are introduced to the lush spring grasses. Previously, it was supposed that it was the protein content in the spring grass which initiated the laminitis. However, recently studies from Australia raised the possibility that the high fructan content in the spring grass might be the reason for the increase of laminitis incidents in the spring.

A low and high fructan variety from DLF-TRIFOLIUM was investigated through the growth season in 2004 and besides illustrating the differences in the fructan content in the two varieties, an increase in the fructan content was measured in both varieties in September.

4. Fructan content in *Lolium perenne*

This chapter contains supplementary data to the results presented in the papers or results which were not included in the papers (chapter 3), but are nevertheless considered sufficiently important to support the findings obtained during this thesis.

In summary, the chapter describes an experiment where the growth period necessary to obtain maximum fructan content was determined. In addition, the differences in the fructan content in plant material harvested above 6 cm or in the area 1-6 cm above ground level were investigated. The biological variation in the WSC content in three plants cloned from the same transgenic line was also examined. The chapter also includes results obtained from experiments performed in order to investigate factors that could influence the amount of fructan synthesised in the transgenic lines. These factors include investigation of combination or the number of FT genes, transcription levels of heterologous and homologous FT genes and Southern blot analyses. Finally, results from cloning of two putative FTs from *L. perenne* and analysis of T1 generation of high-fructan transgenic lines are presented.

4.1 Materials and Methods

4.1.1 Growth conditions

The growth conditions are identical to the ones described in chapter 3.1.

4.1.2 Analysis of water-soluble carbohydrate content

The analyses of water-soluble carbohydrates were performed according to the method described in chapter 3.1.

4.1.3 Transformation of *Lolium perenne*

The constructs used for transformation and transformation protocol are identical with the methods described in chapter 3.1. Information regarding the introduced genes are summarised in table 4.1.

Table 4.1 Overview of the used heterologous genes.

Gene	Construct name[a]	Specie	Accession number	Reference	Primers for cloning	Primers for PCR and RT-PCR analysis[b]
1-FFT	*Cs1-FFT* (O7)	Artichoke (*Cynara scolymus*)	AJ000481	(Hellwege *et al.*, 1998)	Cs1-FFT-fwdC 5'ATGAGAACGACTGAA CCCCAAACTGACC 3' Cs1-FFT-revC 5' CCGGGCTAAAACGGA TAAGCCTTGACAAAGGCTTGAGAC 3'	Cs1-FFT-fwdP 5' AATGCATCCGACCCACTTCTTGTT 3' Cs1-FFT-rev 5' TATCTGGGAACTACTCACACA 3'
1-SST	*Ac1-SST* (K41)	Onion (*Allium cepa*)	AJ006066	(Vijn *et al.*, 1998)	K41-fwdC 5'ATGGAATCCAGAGATATCGAG 3' K41-revC 5' CGGGTCAAGGAGCTG GAATCCGGGGAATGG 3'	K41-fwdP 5'AACGGCAACCCCATCCTCAT 3' K41-revP 5' TATCTGGGAACTACTCACACA 3'
	Cs1-SST (O5)	Artichoke (*Cynara scolymus*)	Y09662	(Hellwege *et al.*, 1996)	Cs1-SST-fwdC 5'ATGGCTTCCTCTACCACCACCCCACTCCTC3' Cs1-SST-revC 5'CCCGGGTCAAGAACTC CATCCAGAAAGAGGG 3'	Cs1-SST-fwdP 5'CTGGGGTGGGATACAAGGATTTTC 3' Cs1-SST-revP 5' TATCTGGGAACTACTCACACA 3'
6G-FFT	*Ac6G-FFT* (K42)	Onion (*Allium cepa*)	Y07838	(Vijn *et al.*, 1997)	K42-fwdC 5'ATGGATGCTCAGGATATTGAGTCCCGTCA 3' K42-revC 5' CCGGGTTAAAAATGAAAAATCATTGT AAGTGGAGTTCATTTGCC 3'	K42-fwdP 5'TGAGGCCACAATAGAAGCAGATG 3' K42-revP 5' TATCTGGGAACTACTCACACA 3'
6-SFT	*Hv6-SFT* (O21)	Barley (*Hordeum vulgare*)	X83233	(Sprenger *et al.*, 1995)	Hv6-SFT-fwd 5' ATGGGGTCACACGGCAAGCCAC 3' Hv6-SFT-rev 5' CCCGGGTTAATGACG AGATTCAAGAACTTGATGAAGATAC 3'	Hv6-SFT-fwdP 5' ACCCCGCCAACCCCGTCATCTG 3' Hv6-SFT-revP 5' TATCTGGGAACTACTCACACA 3'

a: All constructs were made by M. Storgaard, 2002. b: All rev primers are a primer in the terminator part and is therefore similar to all constructs.

4.1.4 DNA isolation and PCR analysis

Isolation of genomic DNA and PCR analysis was performed as described in chapter 3.1. The primers used for confirmation of the genomic integration of the transgenic DNA are listed in table 4.1.

4.1.5 RNA isolation and RT-PCR analysis

Isolation of RNA and RT-PCR analysis was performed as described in chapter 3.1. The primers used for detection of transcription of the introduced genes are listed in table 4.1.

4.1.6 Southern Blot analysis

Genomic DNA was isolated as described in section 3.1 and 20 µg was restricted overnight with 100 units of *Hpa*I and *Pvu*II, respectively (Invitrogen, USA). Similar, the K41 and K42 plasmids were restricted with 10 units of *Hpa*I and *Pvu*II releasing the part of the cassette containing the promoter, GOI and terminator. The DNA samples were fractionated on a 0.8% agarose gel (Invitrogen, USA) and blotted onto Hybond N membrane according to Sambrook *et al*. (1989). The membrane was hybridised with a 420 and 480 bp gene specific DNA fragment from *Ac1-SST* or *Ac6G-FFT*, respectively. The probes were labelled with ^{32}PdCTP (GE Healthcare, UK) using the random primer method (Megaprime, GE Healthcare, UK). Prehybridisation, hybridisation and the subsequent washing steps were performed at 65°C as described in (Sambrook *et al.*, 1989). Signals were detected by exposing the membrane to MS Biomax autoradiography films (Kodak, USA) for two to seven days at -80 ºC and developed at Kodak X-omat, 1000 processor (Kodak, USA).

The membrane was stripped for the probe by washing the membrane in 100 ml 2xSSC + 0.1%SDS at 75ºC for 2x30'. Removal of signal was controlled before next hybridisation was performed.

4.1.7 Thin Layer Chromatography

The material used for thin layer chromatography analysis was extracted as described in chapter 3.1. Aliquots (1 ml) were evaporated to dryness in a Maxi Dry Lyo speed vacuum centrifuge (Heto-Holten, Denmark) and dissolved in 50 µl water. Ten µl were spotted onto 20 x 20 cm aluminium sheets Silica Gel 60 F_{254} and the chromatograms were developed twice at room temperature in a mixture of butan-1-ol:propan-1-ol:water in a ratio of 3:12:4 v/v (Sigma-Aldrich, USA). First run was performed over night, and the next day, the plate was dried with a hairdryer before it was run again for 8 hrs. The chromatograms were made perceptible by spraying with a fructose specific reagent (Wise *et al.*, 1955) and heated for 5 min at 80ºC.

A standard was prepared by adding equal amount of 1 % (v/w) fructose, sucrose, 1-kestose and 1,1-kestotetraose, respectively and run as a mixture. As an alternative standard, 2 g of fresh Danish wild type Chicory was extracted as described under WCS analysis. From both standards, 6 µl were spotted on the chromatograms.

4.1.8 Cloning of putative fructosyltransferases and fructan exohydrolases

Cloning of coding regions of *LpFTa* and *LpFTb* are decribed in chapter 3.1.

Cloning of *LpFEH* was performed using degenerated primers based on different fructan exohydrolase sequences from monocots:

LpFEHdeg-fwd 5'-GCCATGGCMCAAGCTTGGGCCTTCCT-3'

LpFEHdeg-rev 5'-CCAHCCTTTYTYRABRKCATCT-3'.

The template was perennial ryegrass (*Lolium perenne* L, genotype F6, DLF-TRIFOLIUM) grown for 4 weeks at LD and moved to 5°C for five days before the material was harvested for mRNA isolation (described in chapter 3.1).

The RT-PCR reaction of 25 µl contained 0.5 µg cDNA, 0,25 U of Adv2 polymerase, 1x standard reaction buffer 2 (BD science, USA), 3.5 mM $MgCl_2$, 5 nmol dNTP and 0.5 pmol of each primer. Amplification was achieved by denaturation at 94ºC for 2 min followed by 35 cycles of 94ºC for 15 sec, 60ºC for 30 sec and 72ºC for 1 min.

Screening of two different cDNA libraries as previously described (chapter 3.1) resulted in five partial clones and by using the sequences from these clones, new primers were designed

LpFEHfwd 5'-[redacted]-3'

LpFEHrev 5'-[redacted]-3'

The RT-PCR reaction of 25 µl contained 0.5 µg cDNA, 5.0 U of PFU polymerase, 1x standard reaction buffer 2 (Stratagene, USA), 20 nmol dNTP and 0.25 pmol of each primer. Amplification was achieved by denaturation at 94ºC for 45 sec followed by 35 cycles of 94ºC for 45 sec, 57ºC for 45 sec and 72ºC for 5 min using same template as described above.

Neighbour-joining analysis was performed using ClustalW from Megalign, version 5.05 (Lasergene, USA).

4.1.9 Analysis of T1 generation of high fructan transgenic lines

The transgenic lines and the crossing partners were vernalised at 5 ºC for three months before the plants were transferred to summer conditions (16 hrs light at 22 and 20 ºC, day and night temperature, respectively) for floral induction. Each transgenic line was crossed with two crossings partners and were harvested as a mixture after the seeds were matured (*e.g.* the mixture contained the seeds from the transgenic line and one crossing partner).

The seeds were germinated in soil and examined for presence of transgene(s). The transgene T1 lines were also investigated for transgenic transcription using the primers from table 4.1.

4.2 Results and Discussion

4.2.1 Transformation of Lolium perenne with heterologous fructosyltransferases

Besides the transformation of the genes encoding the onion *1-SST* and *6G-FFT*, as reported in chapter 3.1, three other genes encoding FTs from other plant species were transformed into *L. perenne* (table 4.1).

All five genes were combined in six different combinations containing two to four different FT genes in one combination and transformed into *L. perenne*. Table 4.2 gives an overview of the different construct combinations and the number of transgenic lines obtained. The first transformation with *Ac1-SST* and *Ac6G-FFT* was performed in May 2002 and the final transformation with *Ac1-SST*, *Ac6G-FFT* and *Hv6-SFT* in December 2004. As a result of the long time frame from transformation, through screening of transgenic lines (PCR and RT-PCR) to finally growing the RT-PCR positive lines for five months before WSC analysis can be performed, not all the transgenic lines are analysed for fructan content so far. In particular, the five month growth period presented difficulties of keeping the plants in good condition during the summer, where high temperature in the greenhouse increased the use of pesticides. If the plants, used for sugar analysis, were not growing well, they were initiated from one tiller again.

Besides the results presented in chapter 3.1, the highest fructan content (28 % per g DW) was detected in a transgenic line transformed with *Cs1-SST*, *Cs1-FFT* and *Ac6G-FFT*. As previously reported, no correlation between the fructan content and the transcription levels of the heterologous or homologous FTs or the transgene copy number (chapter 3.1) could be observed. The amount of fructans obtained in transgenic perennial ryegrass lines was dependent of the genotypes used for transformation and as illustrated in figure 2 (chapter 3.1). The transgenic lines of the N2 genotype showed, in general, higher fructan levels compared to transgenic lines from the F6 genotype. One possible explanation for this observation is a different morphology, since N2 is characterised by having longer leave sheaths and leaves than F6.

Table 4.2 Overview of the obtained transgenic lines after transformation with the genes encoding heterologous fructosyltransferases.

Combination	Genotype	Number of transformation rounds	Number of petri dishes used for bombardments[a]	Numbers of lines	Number of lines with presence of at least one of the transformed FT[b]	Numbers of lines with transcription of at least one of the transformed FT[c]	Number of lines with high fructan content[d]
Ac1-SST + Ac6G-FFT	N2 and F6	1 and 2	70	65[e]	35 (31)	18 (12)	10 (70 %)[f]
Cs1-SST, Cs1-FFT + Ac6G-FFT	F6	2	57	132	120 (32)	45 (2)	15 (57 %)[f]
Cs1-SST, Cs1-FFT, Ac6G-FFT + Hv6-SFT	F6	3	86	94	30 (3)	20 (0)	2[g] (10 %)[f]
Cs1-SST + Hv6-SFT	F6	1	28	69	56 (28)	14 (6)	3[g] (21 %)[f]
Ac1-SST + Cs1-FFT	F6	2	64	46	n.d.[h]	31 (10)	1[g] (3 %)[f]
Ac1-SST, Ac6G-FFT + Hv6-SFT	F6	4	90	63	n.d.[h]	42 (13)	7[g] (17 %)[f]
Total		**15**	**395**	**469**	**>241 (>94)**	**170 (43)**	**>37**

a: Each Petri dish contain around 30-35 callus for bombardment, b: Number of lines with presence of all the transformed genes are stated in brackets, c: Number of lines with measurable transcription of all the transformed genes are stated in brackets, d: Significantly higher fructan content than the control lines, e: 9 and 56 lines transformed in N2 and F6, genotypes, respectively, f: percentage of transgenic lines with increased fructan content, g: Not all the RT-PCR positive lines are analysed for fructan content and g: the obtained lines from the last transformations were not tested for presence of the transgene(s). All transformations were made by the transformation group at DLF-TRIFOLIUM from May 2002 to December 2004.

Besides the genotype used for transformation, the constructs can also affect the transformation efficiency. As illustrated in table 4.2, the combination of the different constructs gave raise to a different number of transgenic lines. Especially, the transformation with *Ac1*-SST together with *Cs1-FFT* gave the lowest number of antibiotic resistant transgenic lines, with only one containing significantly higher fructan content despite of two rounds of bombardment. This could be a result of poor efficiency of the introduced genes encoding for the FTs but also transformation conditions of the callus affecting the results. The high number of lines with presence of the transgene(s) from the bombardment with *Cs1-SST*, *Cs1-FFT* and *Ac6G-FFT* illustrates high transformation efficiency. Also, high percentage of transgenic lines with increased fructan content could be measured. Only the transformation with *Ac1-SST* and *Ac6G-FFT* gave a higher percentage of high fructan transgenic lines.

In conclusion, the results demonstrated the importance of selecting the best genotype for transformation. However, as table 4.2 also illustrates, it is not always possible to combine high transformation efficiency with improved fructan content.

The subcellular location where the FT is expressed is important for the success of the transgenic approach. For example, a high intracellular concentration of sucrose in the host plants in the correct compartment might be necessary to achieve high fructan levels (Cairns, 2003). The constructs used in this thesis, were not additionally modified in respect to direct the location and as for other transgenic studies, the location of the transformed FTs has not been demonstrated. None of the heterologous FTs, used for transformation in this thesis, contained the vacuolar targeting signal, which could be found in *LpFTa* (section 4.2.4). However, using the heterologous FTs, it was possible to improve the fructan content in transgenic lines with up to 3-fold without inducing unwanted phenotypes.

As fructans accumulate during drought, it has been proposed that they have a role in drought tolerance against (Amiard *et al.*, 2003; De Roover *et al.*, 2000; Thomas and James, 1999). However, this hypothesis has not been supported experimentally. Using a number of perennial transgenic lines transformed with *Ac1-SST* and *Ac6G-FFT* containing different amounts of fructans, the drought tolerance was determined (data not shown). Both the control and transgenic lines were cloned from one tiller and placed in an equal amount of soil. Watering at different time points failed to demonstrate any correlation between fructan content and improved drought tolerance. However, that the tested lines were not grown for five months before they were analysed for drought tolerance, in contrast to previous studies, might provide an explanation for the lacking correlation. Therefore, no conclusive results have been obtained for this hypothesis.

4.2.2 Determination of optimal growth period to obtain maximum fructan content

The optimal growth period to obtain maximum fructan content was investigated in untransformed control plants prior to characterisation of the fructan content in transgenic lines. Two control lines (C1 and C2) and one transgenic plant transformed with a control plasmid (C3) were grown for 7 months under greenhouse conditions. Material was harvested each month starting from month three to seven and the fructan content was measured (figure 4.1).

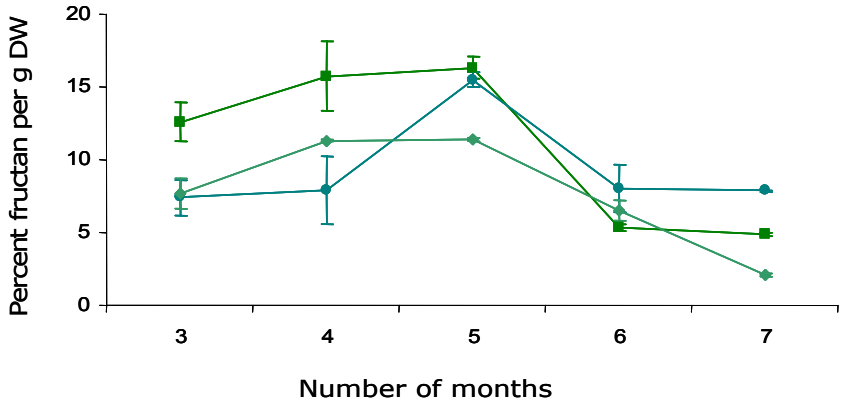

Figure 4.1 Determination of optimal growth period to obtain maximum fructan content.
C1 (—■—) and C2 (—●—) (N2 and F6 genotype, respectively) are non-transgenic lines and C3 (—◆—) is a transgenic line transformed with a control plasmid and were grown for three to seven months under green house conditions. Values are the mean of duplicate extractions ± SE. Where no error bar is included, the error was smaller than the symbol.

The results showed that the maximum fructan content under our greenhouse conditions was measured after four to five months of growth. The fructan content was never higher than 15 % fructan per dry weight. Two of the investigated control lines contained more or less the same fructan content after four or five months of growth, whereas the last control line contained a maximum fructan content after five months of growth. After five months, a decrease in the fructan content could be measured and further decreased or stable low fructan content after seven months of growth was observed. The feature of highest fructan content after 4-5 months was also detected, when in similar experiments transgenic or elite varieties were grown for the same period of time in the greenhouse (chapter 3.1 and data not shown).

The content of glucose, fructose and sucrose did not reach a level higher than 8 % sugar per g DW throughout the investigated growth period (figure 4.2).

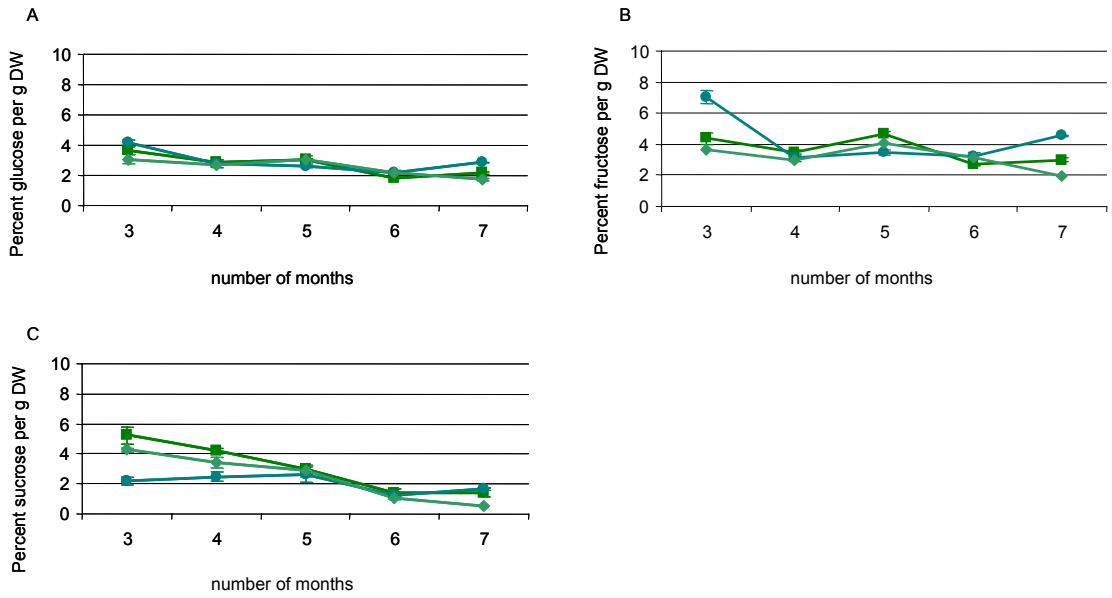

Figure 4.2 Content of glucose, fructose and sucrose through a growth season of three to seven months. C1 (—■—) and C2 (—●—) (N2 and F6 genotype, respectively) are non-transgenic lines and C3 (—◆—) is a transgenic line transformed with a control plasmid. The content of glucose (A), fructose (B) and sucrose (C) were determined after three to seven months growth under green house conditions. Values are the mean of duplicate extractions ± SE. Where no error bar is included, the error was smaller than the symbol.

Since the fructose content did not increase when the fructan content decreased (after five months), there is no indication that the decreased fructan content might be a result of fructan degradation (figure 4.2B). However, the decreasing sucrose content could be the reason for the lack of continuous fructan synthesis after five months (figure 4.2C).

In conclusion, when analysing transgenic *L. perenne* lines grown under our greenhouse conditions, the optimal growth period to obtain maximum fructan content was measured to be four to five months.

One can speculate whether the observed decrease in fructan content in our experiment is a function of a decreased need for fructans in the plant or whether it is a consequence of the fact that the fructan is synthesised in other sink tissue in the plant. Fructans accumulate when photosynthetic carbon supply exceeds demand for growth and development and are stored primarily in the stems and roots, but also the leaves contain fructans (Pavis *et al.*, 2001; Morvan-Bertrand *et al.*, 1999). In our experiment (figure 4.1), only the aerial part of the plant above 6 cm from ground level was analysed, so it is possible that the decreased fructan level measured is a consequence of fructan synthesis in an other sink organ, *i.e.*, the lower part of the stems (1 to 6 cm) or roots of *L. perenne*.

To investigate this hypothesis, the fructan content was determined in the aerial part harvested at 6 cm or at 1-6 cm above the ground level. The results showed that the fructan content in the stubble at 1-6 cm was in average 30 % higher than the fructan level in the aerial part above 6 cm harvested from the ground level. Furthermore, the fructan content did not

increase in the aerial part 1-6 cm from the ground, when the fructan content in the aerial part above 6 cm was decreasing after five months. Therefore, it can be concluded that the decreased fructan content above 6 cm does not lead to increased fructan synthesis or deposition in 1-6 cm part of the stems. However, it can not be excluded that the fructans are synthesised and/or stored in the roots.

A TLC analysis indicated that the fructans from aerial part at 1-6 cm displayed the same DP values as the fructans synthesised in the aerial part above 6 cm from the ground level (data not shown).

In conclusion, this investigation showed that it is very important to standardise the harvest time as well as the plant material harvested in order to achieve reliable fructan content, since the fructan content is higher in the aerial part at 1-6 cm than above 6 cm from the ground level. We decided for our further experiments to grow the plants for five month and to harvest leaf material above 6 cm from ground level.

4.2.3 Estimation of biological variation in the WSC content in three independently plants from four different transgenic lines

To standardise the plants before measuring the WSC content in both control and transgenic lines, the plants were cloned from one tiller and allowed to re-grow for five months before harvesting at 6 cm above the ground level. However, the biological variation between the different tillers cloned from one plant must also be investigated in order to determine a possible influence on variation. Therefore, from four different transgenic lines, three independent clones from each transgenic line were initiated from one tiller each and grown for five months before harvesting. The transgenic lines (12342, 13401, 12595 and 12283) were transformed with *Cs1-SST*, *Cs1-FFT* and *Ac6G-FFT* (see section 4.2.1). One line (12342) apparently did not transcribe any of the transformed genes and was therefore used as control line, whereas 13401 transcribed *Cs1-SST* and *Cs1-FFT*, 12595 Cs1-FFT and 12283 *Ac6G-FFT*, respectively.

From each plant, the WSCs were extracted twice and the measured WSC content showed that not unexpected a biological variation was present in the analysed transgenic lines (figure 4.3).

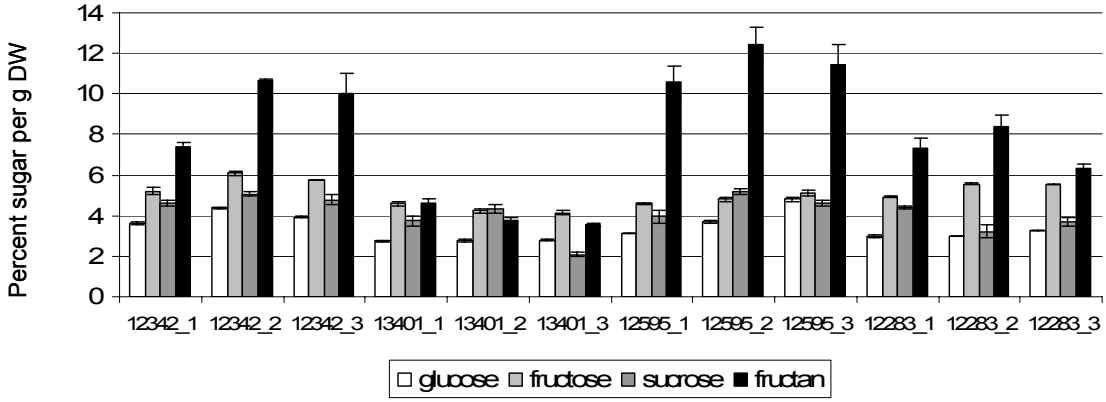

Figure 4.3 Biological variations in the content of water soluble carbohydrate in four independent transgenic lines.
The transgenic lines (12342, 13401, 12595 and 12283) were transformed with *Cs1-SST*, *Cs1-FFT* and *Ac6G-FFT* (chapter 4.4). One line (12342) was not positive for transcription of none of the transformed genes, whereas the other lines (13401, 12595 and 12283) were positive for *Cs1-SST* + *Cs1-FFT*, *Cs1-FFT* and *Cs1-FFT* and *Ac6G-FFT*, respectively. Values are the mean of duplicate extractions ± SE. Where no error bar is included, the error was smaller than the symbol.

For the four different sugars measured in the transgenic lines, it could be determined that a certain variation in the sugar content of three independent clones from each transgenic line is present. The three clones from the control line contained between 7.5 to 10.5 % fructan per g DW, whereas the transgenic lines (13401, 12595 and 12283) contained 3.8-4.7, 10.5-12.3 and 6.2-8.3, respectively. Analysing the data with 95 % confidence intervals, not all of the measurements could be found within this interval. Despite the low number of repetitions, the results at least indicate that a biological variation in one tiller cloned plants can not be excluded.

Finally, it can be concluded that, since the time point, where the control and the transgenic lines are leaving the containers after tissue culture are very different, and since it is known that fructan content is depending on the growth period, it is necessary to synchronise the lines before analysing. Therefore, the control and transgenic lines were still cloned from one tiller in order to make the lines more comparable despite of the biological variation, and only transgenic lines with significant higher fructan content (minimum at 95% probability), compared to the maximum level of fructans in control lines (15 %, chapter 3.1), were classified as high fructan transgenic lines.

4.2.4 Determination of transgene copy numbers

Factors like the transcription levels and/or the copy numbers of the integrated genes can influence fructan content in the transgenic lines. The data from the analyses of transcription levels are presented in chapter 3.1, where also the data from Southern blot analysis are mentioned but not shown. In this section the results are described and illustrated.

Six transgenic lines, transformed with *Ac1-SST* (K41) and *Ac6G-FFT* (K42) and with different fructan levels, were analysed in a Southern blot analysis. K41 and K42 were

restricted with *Hpa*I and *Pvu*II, resulting in fragments containing the promoter, GOI and terminator (figure 4.4).

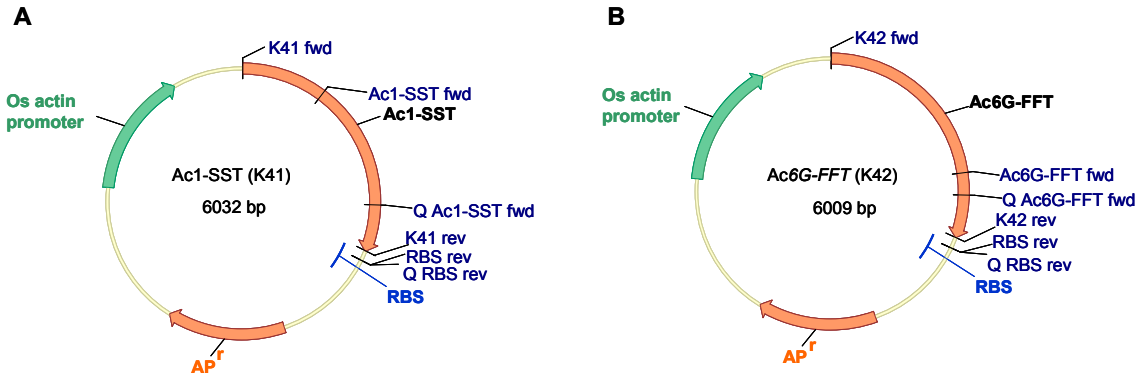

Figure 4.4 Plasmid maps of *Ac1-SST* (K41) and *Ac6G-FFT* (K42).
Restriction sites for enzymes used for restriction of the genomic DNA or for preparing probes are illustrated. The red arrow indicates where the probes hybridised to in the constructs. Resistance to ampicillin is marked as APR and RBS marked the position of the terminator. A: *Ac1-SST* (K41) and B: *Ac6G-FFT* (K42).

For designing gene specific probes, the coding sequence of *Ac1-SST* and *Ac6G-FFT* were aligned to each other and the area with lowest homology (*e.g.* the area between 450 and 950 bp) was selected for designing probes (Figure 4.5).

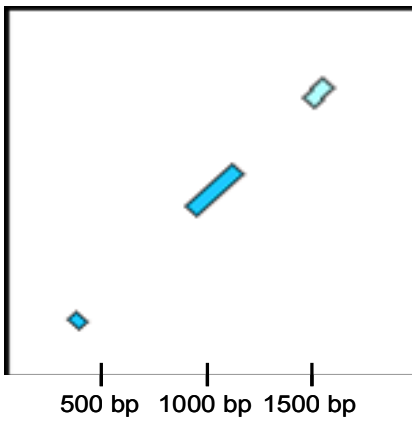

Figure 4.5 Alignment of *Ac1-SST* and *Ac6G-FFT*.
The nucleotide sequence of Ac1-SST (accession number AJ006066) and Ac6G-FFT (accession number Y07838) were aligned using bl2seq (NCBI, 2006). In the first small dark blue area with homology, the sequence identity was 84%, whereas

K41 and K42 were restricted with 10 units/µl of *BamH*I + *Kpn*I and *Pml*I + *Sal*I giving a fragment from each construct on 465bp and 420 bp, respectively (figure 4.5). These fragments were labelled radioactively and hybridised onto the Southern membranes.

The membrane was probed first with the K41 fragment labelled with 32P-labelled dCTP and the results showed that the transgenic lines contain five to eight copies of K41 (figure 4.6 and table 4.3).

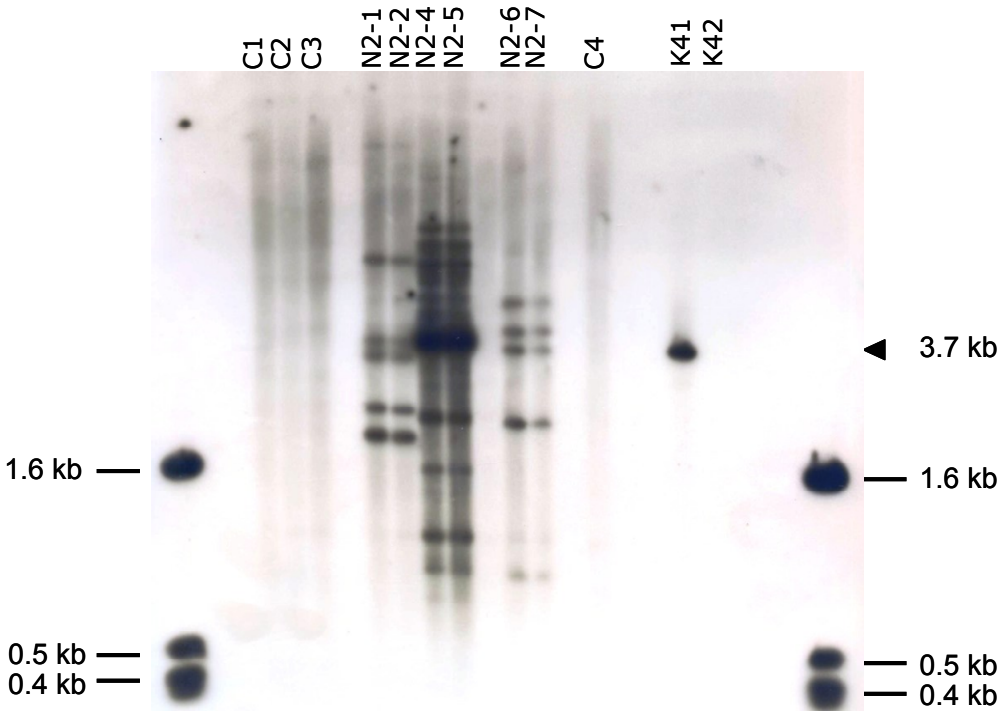

Figure 4.6 Determination of *Ac1-SST* copy number.
Southern blot analysis of selected transgenic lines with different amount of fructan content. Genomic DNA was restricted with *Hpa*I and *Pvu*II releasing a 3.7 kb fragment containing the promoter, *Ac1-SST* and terminator (arrowhead) and probed with 0.42 kb *Ac1-SST* fragment. C1 and C2 are non-transgenic lines (N2 and F6 genotypes, respectively. C3 is transformed with a control plasmid and C4 with other heterologous fructosyltransferase genes, respectively. N2-1, N2-2, N2-4, N2-5, N2-6 and N2-7 are all transformed with *Ac1-SST* and *Ac6G-FFT*.

The transgenic lines N2-1, N2-2, N2-6 and N2-7 contained a fragment of the expected size (3.7 kb), indicating an integration of the promoter, GOI and terminator. However, in all transgenic lines, additional fragments larger than 3.7 kb could be seen, indicating that the cassette was integrated in different areas of the genome. Fragments smaller than 3.7 kb, indicate that integration in the genome has, in some cases, resulted in disruption of the cassette and only minor parts of the cassettes were integrated. However, from all the selected transgenic lines, detection of a RT-PCR product spanning the 3' end of the two integrated GOI and the terminator was obtained indicating measurable transcription of both introduced genes.

The membrane was stripped for K41 probe before hybridisation with K42 was performed.

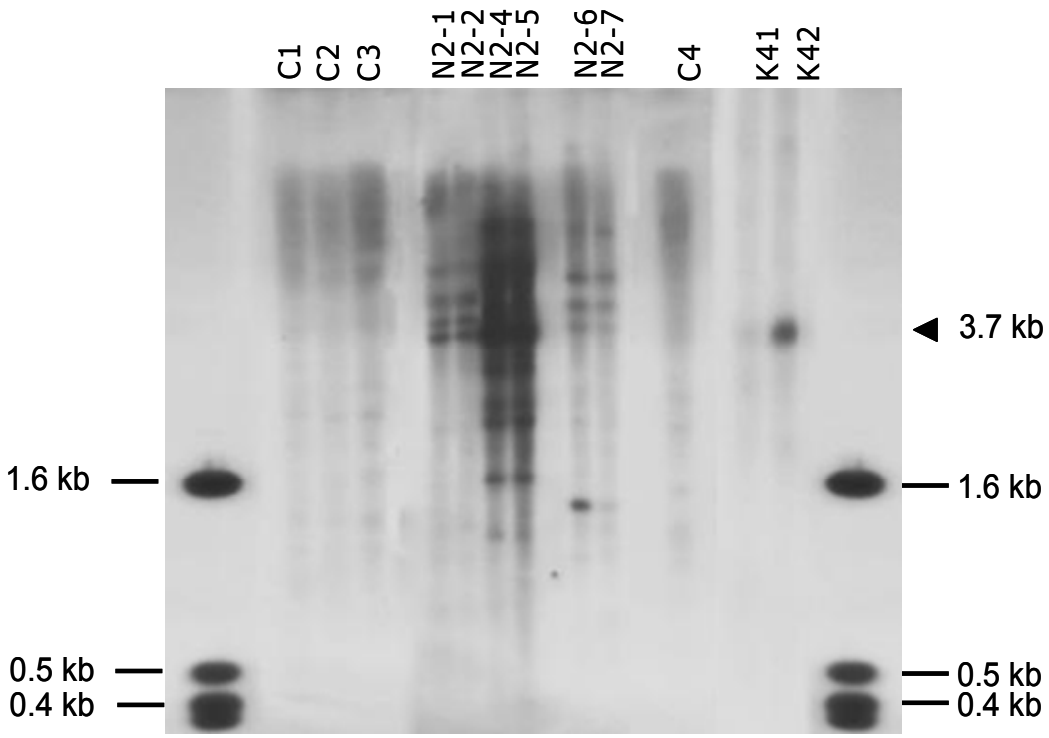

Figure 4.7 Determination of *Ac6G-FFT* copy number.
Southern blot analysis of selected transgenic lines with different amount of fructan content. Genomic DNA was restricted with *Hpa*I and *Pvu*II releasing a 2.3 kb fragment containing the promoter, *Ac6G-FFT* and terminator (arrowhead) and probed with 0.47 kb *Ac1-SST* fragment. C1 and C2 are non-transgenic lines (N2 and F6 genotypes, respectively). C3 is transformed with a control plasmid and and C4 with other heterologous fructosyltransferase genes, respectively. N2-1, N2-2, N2-4, N2-5, N2-6 and N2-7 are all transformed with *Ac1-SST* and *Ac6G-FFT*.

The results showed that the transgenic lines contain at least four to nine copies of K42 (Figure 4.7 and table 4.3). Only the transgenic lines, N2-6 and N2-7 contained a fragment of the expected size (3.7 kb) indicating an integration of the cassette covering the promoter, GOI and terminator.

Table 4.3 Estimated numbers of transgenic copies.

Transgenic line	Percent fructan content/g DW	Number of *Ac1-SST* (K41) copies	Number of *Ac6G-FFT* (K42) copies
N2-1	19.17	5	4
N2-2	18.90	5	4
N2-4	40.81	8	9
N2-5	23.55	8	9
N2-6	28.04	5	5
N2-7	28.11	5	4

Transformation of *Ac1-SST* and *Ac6G-FFT* results in transgenic lines with multiple copies (four to nine). But, the fructan content could not be correlated to the copy numbers, since transgenic lines with 20 or 28 % fructan/g DW both contained four to five copies of *Ac1-SST* and *Ac6G-FFT* (table 4.3). Therefore the difference in the fructan content could not be correlated to the number of transgene copies.

4.2.5 Cloning of putative fructosyltransferases and fructan exohydrolases

As described in chapter 3.1, two genes encoding putative fructosyltransferase from *L. perenne* were isolated by screening cDNA libraries. The isolated genes were designated *LpFTa* (accession number DQ408726) and *LpFTb* (accession number DQ408727).

Alignment to fructosyltransferases from other monocots shows that *LpFTa* is more related to *1-SST*s (figure 2.4 in section 2.2.3) indicating that *LpFTa* may possess 1-SST activity. *LpFTa* shows between 94-99 % sequences identities at amino acid level when comparing to other 1-SSTs from grasses (*Lp1-SST* and *Fa1-SST*, accession numbers AY2454531 and AJ297369, respectively). It was observed that *LpFTa* in one case (position 106) contained an amino acid which was not identical to the other 1-SST from *L. perenne* but was identical to 1-SST from *F. arundinacea* (appendix 6.3), which could be a result of a different allele.

The other gene encoding a putative FT, *LpFTb*, displays highest similarity to a group of putative fructosyltransferases with uncharacterised enzymatic activities (figure 2.4 in section 2.2.3). The closest functionally characterised fructosyltransferase with homology to *LpFTb* is *6-SFT* from barley, which might indicate that *LpFTb* displays 6-SFT-like activity. The homology to the other *L. perenne* genes from the same group has 96-99% sequence identities and only in one position (31), *LpFTb* and *Lp6-FT* differ from the other gene from *L. perenne* and *L. temulentum* (appendix 6.4), which could indicate a different allele.

It has been suggested that FTs have evolved from vacuolar invertases (Vijn and Smeekens, 1999). Previously, a vacuolar targeting signal sequence [R(G)(V)XXGVS(E/D/Q)K(S,T,A)] for vacuolar invertases was identified, but this sequence was lacking in the FTs investigated (Balk and de Boer, 1999). Interestingly, the vacuolar signal sequence can be found in *LpFTa* and the 1-SSTs homologous to *LpFTa* and also in the genes encoding for 6G-FFT activity. However, *LpFTb* and the FTs most identical to *LpFTb* do not contain this vacuolar targeting signal sequence (figure 4.8).

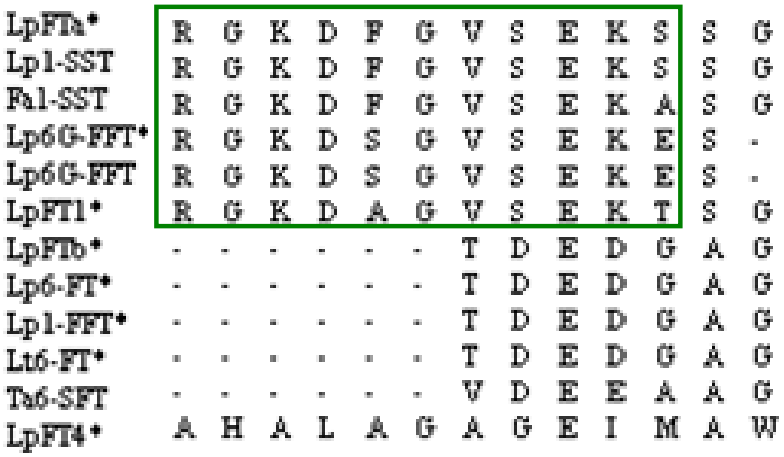

Figure 4.8 Comparison of the vacuolar targeting signal domain of the deduced amino acid sequence of *LpFTa* and *LpFTb* aligned to genes encoding fructosyltransferases from other monocots.
The green box illustrates the vacuolar targeting signal sequence. The accession numbers are: *L. perenne LpFTa**, DQ408726; *L. perenne Lp1-SST*, AY245431; *F. arundinacea Fa1-SST*, AJ297369; *L. perenne Lp6G-FFT** AB125218; *L. perenne Lp6G-FFT* AF492836; *L. perenne LpFT1** AF481763; *L. perenne LpFTb**, DQ408727; *L. perenne Lp6-FT**, AF494041; *L. perenne Lp1-FFT**, AB186920; *L. temulentum Lt6-FT**, AJ532550; *T. aestivum Ta6-SFT* AB029887 and *L. perenne LpFT4**, DQ073970. The names marked with * is uncharacterised genes.

This could indicate that these fructosyltransferases are present in other cellular compartments than the vacuole or even that they are extra-cellular. Using a prediction database (Xie *et al.*, 2005), the localisation of *LpFTa* was predicted to be in the vacuole and *LpFTb* to be extra-cellular, supporting this hypothesis.

Besides cloning of two putative FTs, also genes encoding putative FEHs were cloned. Sequence comparison of different clones encoding fructan exohydrolase from monocots was the basis of design of degenerated primers. Several fragments were obtained and one of them showed 83 % sequence identities to 1-FEH from barley (accession number AJ605333) (Nagaraj *et al.*, 2004). This fragment was used for screening of our *L. perenne* cDNA libraries. Screening of the first library, constructed from vegetative growing plants (chapter 3.1), resulted in three positive clones, but unfortunately none of them contained the 5' end of the coding sequence. Despite several attempts to clone the 5' end using the 5' RACE technique, no full-length clones could be obtained.

Screening of another cDNA library, constructed from vernalised plants (chapter 3.1) resulted in the isolation of further two clones. Again, the gene fragments were lacking parts of the coding region resulting in premature stop-codons. However, using the gathering sequence informations, generated from the cloning of the 5' and 3' partial cDNA sequences, new primers were designed and a full-length fructan exohydrolase (*LpFEHa*) clone was obtained in a RT-PCR based approach. *LpFEHa* showed 82 % sequence identity to an uncharacterised putative FEH from *L. perenne* (accession number DQ073968) and 79 % sequence identity to

a characterised 1-FEH from wheat (accession number AJ508387) (Chalmers *et al.*, 2005; Van den Ende *et al.*, 2003).

Using the same primers as for cloning of *LpFEHa*, other putative FEHs were cloned using different templates (table 4.4).

Table 4.4 Overview of the putative *LpFEHs* cloned from different origins and with different sizes.

	Template	Size (bp)
LpFEHa	cold induced F6 leaves	1596
LpFEHb	sucrose induced F6 leaves	1743
LpFEHc	sucrose induced F6 leaves sheaths	1743
LpFEHd	sucrose induced N2 leaves sheaths	1743
LpFEHe	F6 sheath leaves	918*
LpFEHf	N2 sheath leaves	915*

*: Spliced versions of FEHs.

As listed in table 4.4, *LpFEHe* and *LpFEHf* were around half the size of de other isolated FEHs and are probably spliced versions and based on sequence identities, the clones were annotated *LpFEHs*. Comparing the sequences of *LpFEHe* and *LpFEHf*, it could be observed that within the first 58 amino acids, *LpFEHe* and *LpFEHf* align 100 % to *LpFEHa*, subsequently followed by a spliced region between position 59-335 in *LpFEHe* and *LpFEHf* (figure 4.9).

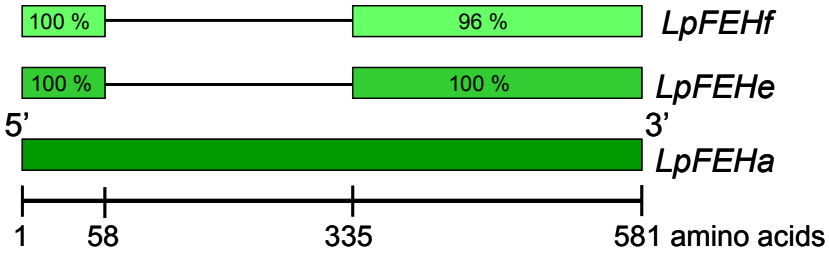

Figure 4.9 Sequence comparisons of *LpFEHa, LpFEHe* and *LpFEHf*. The deduced amino acids of the isolated clones were aligned and the percentage marked in the boxes represents the sequence identities of *LpFEHe* and *LpFEHf* compared to *LpFEHa*.

After amino acid 336, the sequence of *LpFEHe* and *LpFEHf* align again with 96 or 100 % sequence homology to *LpFEHa*, respectively. This indicates that *LpFEHe* and *LpFEHf* are small versions of *LpFEHa* containing an ORF of 306 and 305 amino acids, respectively.

Analysing the cDNA clones for three well conserved regions of FTs and FEHs, it could be observed that only the NDPNG motif was present in all the isolated clones, whereas the

FRDP and WECPD motifs were absent in the small versions of FEHS (*LpFEHe* and *LpFEHf*) (figure 4.10).

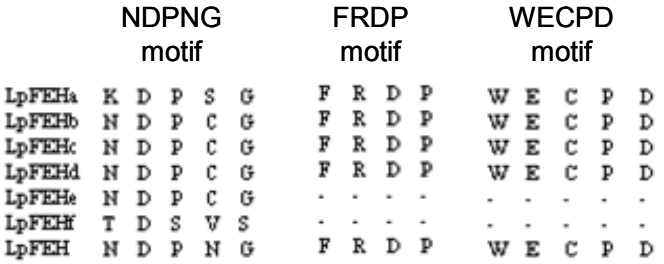

Figure 4.10 Alignment of the deduced amino acid sequence of three well conserved regions of FEHs. LpFEHa-LpFEHf represents the isolated clones during the PhD thesis and has no accession numbers. LpFEH represents L. perenne FEH (DQ073968).

Despite that all the FEH clones contained the NDPNG motif differences were observed. However, from FTs it is well known that differences in the NDPNG motif occur. *e.g.* in *LpFT4* (accession number DQ073970), where the sequence is SDPSG (Chalmers *et al.*, 2005). Opposite, the FRDP and WECPD motifs were 100 % conserved in *LpFEHa*, *LpFEHb, LpFEHc, LpFEHd* and *LpFEH* (figure 4.10).

Besides the cloning of the cDNA clones, genomic clones from N2 and F6 genotypes were also isolated. The obtained clones showed 96 % sequence homology to each other and contained both of five exons. Previously, it was reported that the genomic sequence of two different FTs of *L. perenne* contained four exons including a 9 bp mini exon (Chalmers *et al.*, 2005). This mini exon was not present in any of the isolated genomic clones and as it has neither been reported for the *1-SST* gene from *L. perenne* and *F. arundinacea* (Chalmers *et al.*, 2003; Luscher *et al.*, 2000).The lack of this mini exon could indicate an existence of an alternative splicing under certain physiological conditions as it has been reported for an acid invertase from potato, which was exposed to cold stress (Chalmers *et al.*, 2005; Bournay *et al.*, 1996).

It can be concluded that based on sequence identities, several or maybe all of the isolated FEHs in this thesis could represent new FEHs. However, sequence homology does not always allow classification of newly isolated genes. It was recently reported that a FT gene with 81 % sequence homology to 1-SST from same species, did not encoded the same enzyme activity and the gene was therefore renamed after functional characterisation (Lasseur *et al.*, 2006). Therefore, the genes must be functional analysed, before it is possible to predict the relative enzyme function or whether the smaller splice versions (*LpFEHe* and *LpFEHf*) encodes for a functional enzymes.

4.2.6 Analysis of T1 generation of high fructan transgenic lines

To investigate the possibility to transfer the high fructan trait to the next generation, selected transgenic lines were crossed into two different DLF TRIFOLIUM elite varieties (Indiana and Cancan). Both varieties were analysed for fructan content under the same greenhouse conditions as the T1 generation and the maximal fructan content measured after five months of growth never exceeded 15 % per g DW (chapter 3.1).

The analysis of T1 generation is illustrated in table 4.5 and 4.6, respectively. The high percentage of transgenicT1 lines is a result of multiple copies of the transgene integrated in the T0 lines as previously determined (section 4.2.4).

Tabel 4.5 Results from crossing of transgenic lines with a N2 genotype.

	Numbers of T0 lines	Numbers of T1 lines	Number of T1 lines with presence of at least one of the transformed FT	Numbers of T1 lines with transcription of at least one of the transformed FT	Number of T1 lines with high fructan content
Ac1-SST* + *Ac6G-FFT	9	1728	30	7	1

Tabel 4.6 Results from crossing of transgenic lines with a F6 genotype.

	Numbers of T0 lines	Numbers of T1 lines	Number of T1 lines with presence of at least one of the transformed FT	Numbers of T1 lines with transcription of at least one of the transformed FT[a]	Number of T1 lines with high fructan content
Ac1-SST* + *Ac6G-FFT	3	537	247	84 (30)	23
Cs1-SST*, *Cs1-FFT* + *Ac6G-FFT	8	1116	496	307 (80)	11

a: Only five T1 lines from each crossing were used for determination of fructan content.

The high number of T1 lines is a consequence of the fact that the seeds were harvested as a mixture of the T0 line and the crossing partner (table 4.5 and 4.6). In order to investigate whether the co-bombarded selection marker could be crossed out, the T1 lines were not selected with antibiotic.

There was a remarkable difference between the numbers of T1 lines containing at least one of the transgenic FTs comparing the two genotypes with N2 genotype being less effective. As the same crossing partners were used for crossing of both T0 transgenic lines with N2 or F6 genotypes, the lack of positive T1 lines can not be explained by the crossing partners, since many transgenic T1 lines were generated in the crosses with F6 genotype. Transgenic lines with N2 genotype have not been crossed before neither it has been used as crossing partner.

The following year, the cross with the nine high fructan transgenic lines in N2 genotype was repeated. Only two out of the nine T0 transgenic lines produced flower stems and none of the seeds were able to germinate. This might indicates difficulties with reproduction of next generation in transgenic lines with N2 genotype, which could be N2 genotype specific, a result of transformation procedure or the high fructan trait. However, it is unlikely that the high fructan trait should be the reason for lack of transgenic T1 lines with N2 genotype since it has been possible to transfer the high fructan content of 25 % in the F6 genotype to the next generation. Since some of the transgenic lines in N2 genotype contain fructan contents below 25 % per g DW, it is unlike that the difficulties to obtain transgenic T1 lines with N2 genotype should be a result of the high fructan trait.

In order to perform a further investigation of the problems with the N2 genotype, wild type plants from the N2 genotype have also been vernalised this winter and will be crossed with high fructan transgenic T0 lines of the F6 genotype in order to evaluate the possibility of transferring the high fructan trait into the N2 genotype.

From the number of transgenic T1 lines with transcription, only five T1 lines from each crossing were further analysed for fructan content. Transgenic T1 lines with similar or higher fructan content than the T0 line were scored as T1 lines with high fructan (table 4.3, 4.4 and figure 4.11).

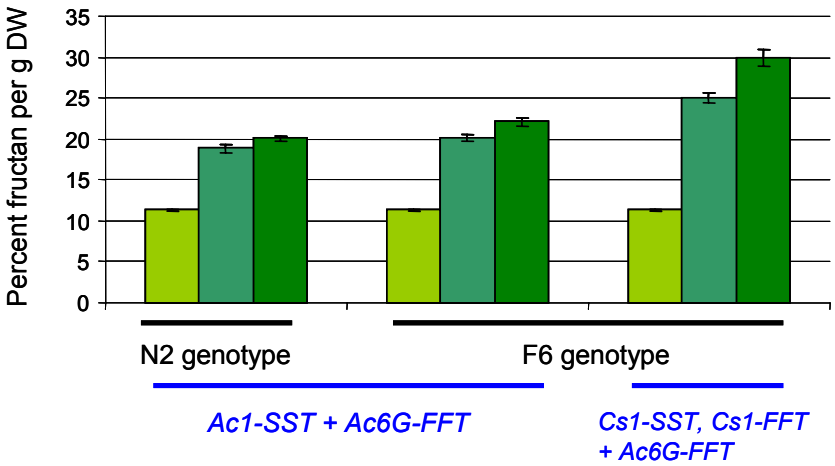

Figure 4.11 Fructan content in the crossing partner, T0 and T1 line from each genotype or combination of FTs.
The N2 genotype was transformed with *Ac1-SST* + *Ac6G-FFT*, whereas the F6 genotype was transformed with *Ac1-SST* + *Ac6G-FFT* or *Cs1-SST*, *Cs1-FFT* + *Ac6G-FFT*. ■, ■ and ■ represents the fructan content in crossing partner (Cancan), T0 and T1 lines, respectively.

No data on T1 generation from high fructan transgenic perennial ryegrass lines have been reported yet, but a study of crosses between to *L. perenne* varieties has been performed (Humphreys, 1989). The two T0 varieties contained between 19.9 and 24.2 % WSC (an average for a plot experiment) and by growing two T1 plants out of this cross in similar plots experiments, the average WSC was between 21.7 and 25.5 demonstrating a small increase in the WSC content in the T1 generation.

Finally, it can be concluded that transfer of high fructan trait from T0 generation to next generation is possible. For the crossing of transgenic lines in F6 genotype up to 44 % of the T1 seeds contained at least one of the introduced genes and 62 % of the transgenic T1 lines did also transcribed the genes. Despite that only a minor part of the transgenic T1 lines were analysed for fructan content, several transgenic T1 lines contained similar or even higher fructan content.

5. Conclusions and perspectives

Carbohydrates are a fundamental component in all animal feed and in the ruminants. The conversion of nitrogen is a microbial process, which takes place in the foregut. If the supply of readily available energy is abundant, it is vital for the microbial growth and thereby also affecting the ruminants' nutrition (Humphreys *et al.*, 2006).

Many features about the fructan metabolism in *L. perenne* as well as in other plant species are already known but still many factors need to be discovered. As one example, the location of the fructan metabolism can be mentioned. It has been demonstrated, in this PhD thesis (chapter 3.1) and in several other publications (Pilon-Smits *et al.*, 1995; Ebskamp *et al.*, 1994; van der Meer *et al.*, 1994) that it is possible to increase the fructan content using transgenic approaches, but has still to discovered, where the fructan metabolism is takes place. This will not be an easy task, as it so far has not been possible to extract the different kinds of vacuoles which are present in the plant cell.

Another important element of the fructan metabolism is the degradation of fructans performed by FEHs. Despite that several different FEH activities have been reported from *Lolium* species (Marx *et al.*, 1997; Bonnett and Simpson, 1995), only one gene encoding a 1-FEH has been reported cloned (Chalmers *et al.*, 2005) and the work in this PhD thesis revealed the isolation of six putative FEHs. No functional characterisation has yet been reported for any of these genes and therefore the characterisation will provide valuable information about the role of FEHs in the fructan metabolism. It can not be excluded that the FEHs play an extensive role in the fructan metabolism and could thereby be another tool for increasing the fructan content in plants.

An important issue for the improvement of grasses used as fodder in intensive agriculture, is the potential to increase the carbohydrate (*e.g.* fructan) content resulting in enhanced nutritional value, which can improve the milk and meat production (Moorby *et al.*, 2006; Miller *et al.*, 2001). As an additional and important effect of this, the release of ammonia to the environment will decreased, when the fodder is better utilised in the rumen as a result of higher carbohydrate content (Lee *et al.*, 2002). Therefore, the objectives for this PhD project were:

I) Improvement of the fructan content in transgenic *L. perenne* by over-expression of heterologous FT genes
II) Seasonal changes of the water-soluble carbohydrates (WSC) content in DLF-TRIFOLIUMs elite varieties
III) Cloning and molecular analysis of genes encoding FTs or FEHs isolated from *L. perenne*

Improved fructan content in transgenic perennial ryegrass

Preliminary experiments during this PhD project demonstrated that the fructan content at a given time in *L. perenne* is the result of dynamic processes. It was therefore essential to define and standardise the growth conditions as well as the harvest material for the WSC analysis in order to achieve stable and comparable measurements on the parameters affecting the fructan content. These parameters have not been published for *L. perenne* or any other plant species even though several studies of transgenic plants transformed with bacterial or plant fructan synthesising genes have been published. It is therefore difficult to compare the fructan levels achieved during this PhD project with other studies, despite another study of *L. perenne* lines transformed with FTs genes from wheat has been performed (Hisano *et al.*, 2004a). But under the assumption that fresh ryegrass plant material contains around 75 % water, the fructan concentration can be estimated to be around 17 % per g DW in the highest transgenic line from their study compared to 41 % in the investigation during this PhD project. The lower fructan content in their study could be a result of differences in harvesting time, harvesting material and/or the effect of the introduced wheat genes.

In order to perform the investigations of the first objective of this PhD project, five heterologous fructosyltransferases from different plant species were transformed into *L. perenne* in six different combinations and in two different *Lolium* genotypes. In total, more than 450 transgenic lines were obtained and analysed. The highest fructan content achieved was 41 % per g DW, which is more than 3-fold improvement compared to the levels in the control lines. Furthermore, the fructans in the transgenic lines contained higher DP values than in the control lines demonstrating the action of the heterologous genes. As a further advantage of the transgenic approach, the fructan content was demonstrated to be high and stable throughout the growth period. This improvement could not be seen in a traditionally bred variety, marketed as high sugar variety, which was included in the investigation for comparison.

Despite the very high fructan content achieved in the transgenic lines, neither a correlation between the fructan content and the transcription levels nor between transgene copy numbers of the heterologous FTs could be detected. An analysis of transcription levels of two putative FT genes from *L. perenne* itself could not be correlated to the fructan content either. Nevertheless, transgene transcription was a prerequisite to obtain high fructan content since the fructan content never reached more than 15 % per g DW in any of the control lines measured incl. elite varieties grown under our greenhouse conditions.

Besides the possible explanations for the lack of correlation between the fructan content and the investigated factors described above and in the submitted paper, it would be interesting to measure the transcription level of the newly classified FT gene (6G-FFT) (Lasseur *et al.*, 2006) in the transgenic lines.

Another important aspect was to evaluate the possibility of transferring the high fructan trait into the next generations. The results from this PhD project showed that is was possible to transfer the high

fructan trait from two different gene combinations of the heterologous FTs and from both genotypes to the next generation. The F6 genotype gave higher number of transgenic T1 lines and several of them displayed high fructan content. The N2 genotype showed to be difficult to be crossed. The first attempt to generate transgenic T1 lines resulted in only a low number of T1 lines with presence of at least one of the introduced genes. Only 23 % showed transcription of at least one of the transformed genes and 14 % of them showed high fructan content.

The second attempt to cross the transgenic lines with N2 genotype in the following year resulted in only two out of nine flowering transgenic lines and the seeds obtained from these two transgenic lines did not germinate. In a currently running third attempt to generate transgenic T1 lines, the transgenic T0 lines were exposed to a longer vernalisation period and are currently growing at 22 ºC for 16 hrs followed by 8 hrs of 20 ºC.

The difficulties of crossing transgenic lines with N2 genotype could besides of being a result of the N2 genotype, also be an effect of the transformation process (callus phase), but also the high fructan trait could be responsible for this. However, the transfer of a fructan content of 25 % in the F6 genotype has been possible and since the transgenic lines in N2 genotype contain between 19 and 41 % fructan content per g DW, it is unlikely that the difficulties of obtaining a next generation of transgenic T1 lines with N2 genotype might be a result of the high fructan trait. Wild type plants from the N2 genotype have also been vernalised this winter and will be crossed with high fructan transgenic T0 lines of the F6 genotype in order to evaluate the possibility of transferring the high fructan trait into the N2 genotype.

Future work including the high fructan transgenic lines should assess further aspects of the high fructan content. Drought resistance, refoliation rate and winter hardiness are important factors and could open for other aspects to investigate the high fructan lines for. It is possible that the high fructan content is synthesised on behalf of other components in the transgenic lines even though that it has been reported that *L. perenne* under certain growth conditions can contain up to 40 % WSC/fructan per g DW. It could therefore be interesting to investigate other components, which are important to achieve high fodder values.

Also identification of the fructans synthesised in the transgenic lines could be an interesting investigation using methods as HPLC etc.

Analysis of elite varieties from a field experiment

The second objective of this PhD project was to perform an analysis of seven selected elite varieties through two growth seasons, 2004 and 2005. Within each growth season, the elite varieties were analysed from two different establishment years. Based on the obtained results, it can be concluded that independently of the growth season, the WSC and fructan content decreases with increasing

grass sward age as previously reported (Turner *et al*., 2005; Taweel *et al*., 2005; Wilman *et al*., 1996).

The two different growth seasons were characterised by having a different amount of rain fall. Through May to November (2005) it rained 32 % less compared to the same period in 2004. Especially in August 2005, the amount of rain was considerable lower than in August 2004. This was correlated with an increased accumulation of fructans represented as percentage of fructan per total amount of WSC. This increase in percentage could indicate that the fructan accumulation is correlated to drought response as it has been suggested previously (Amiard *et al.*, 2003; Wilson and Kachman, 2001; De Roover *et al.*, 2000; Thomas and James, 1999).

Despite of the differences in the two growth seasons, several of the varieties displayed a WSC/fructan pattern, which was consistent through both growth seasons. Indiana was the variety with the lowest average WSC/fructan content through the growth seasons and is therefore favourable for grazing of horses and ponies, as it is believed that high WSC/fructan content increase the possibility of induction of laminitis (Bailey *et al.*, 2004). The advantage of the low WSC/fructan content has resulted in production of a new grass mixture in Denmark; suitable for grazing of horses and ponies (HorseMax 50S, www.dlf.dk).

Varieties, as Cancan and Falstaff, contained high WSC/fructan content in both growth seasons, which makes them especially useful for grazing of dairy cows as high WSC/fructan content has been reported to increase the milk and meat production (Miller *et al.*, 2001).
The varieties were established in 2003, analysed for WSC content in the growth season 2004 and repeated again in 2005. Thereby, the varieties from same establishment year were analysed twice but with different sward ages. A drawback in this study was that no replicates could be performed in the same establishment years as a result of the fixed set up of the different plots, which were available for this field study. Replicates would have strengthened the statistical analysis of the data from the field experiments and thereby the conclusion of the study.

To verify the obtained results from growth season 2004 and 2005, the experiments will continue for the next years under the same experiment conditions by DLF-TRIFOLIUM.

Genes encoding fructosyltransferases and fructan exohydrolases

In order to fulfil the least objective in this PhD study, two putative FTs (*LpFTa* and *LpFTb*) and six putative FEHs (*LpFEHa-f*) were isolated. Based on sequence identities, the *LpFTa* is likely to encode a 1-SST enzyme and represents probably the same gene as *1-SST* from *L. perenne* (accession number AY245431). This enzyme has been functional characterised (Chalmers *et al.*, 2005) and shares 99 % sequence identity to *LpFTa*.

LpFTb does also share 99 % sequence identity to another gene isolated from *L. perenne* (accession number AF494041) and is therefore probably the same gene. However, none of the *L. perenne* genes, which share high sequence identities to *LpFTb* have been functional analysed. The

gene functional characterisation and with the highest sequence identity (78 %) is 6-SFT from wheat (AB029887). This low sequence homology does however not allow to characterize LpFTb as a 6-SFT. Since the enzyme responsible for the production of the fructan class levan neoseries in *Lolium* remains to be identified, it could be interesting to perform a functional analysis of *LpFTb* in order to determine whether the *LpFTb* gene encodes for the enzyme responsible for synthesis of fructans of levan neoseries.

Recently the cloning of a Lolium gene with 1-FFT activity was reported through personal communication with the scientists, which have cloned the *Lp1-FFT* gene (Chalmers et al. 2005). This gene shares 99% sequence identity with *LpFTb* and aligns together with other *L. perenne* genes, which have been annotated 6-FT. However, the results from the enzyme characterisation have never been published and after submission to the NCBI database, this 1-FFT has been renamed to a putative fructosyltransferease1 (accession number AB186920) making it questionable to believe the reported enzyme activity. It is therefore still possible that *LpFTb* and the other *6-FT*-like genes, which have been isolated from *L. perenne* truly encodes for a 6-XFT enzyme, which could be the enzyme responsible for synthesis of the levan neoseries.

Furthermore, recently it was reported that a gene isolated from *L. perenne* codes for an enzyme with 6G-FFT/1-FFT activity (Lasseur *et al.*, 2006) and together with the gene encoding for the 1-SST enzyme (Chalmers *et al.*, 2003), these two genes can explain the synthesis of two fructan classes in *L. perenne*; Inulin and Inulin neoseries. The 1-FFT-like gene from *L. perenne* (accession number AB186920) shares only 78 % sequence identities to *Lp6G-FFT* (accession number AF492836) which also supports that *Lp1-FFT* does not display 1-FFT activity. However, cautions should be taken to only address the enzyme activity based on sequence identities, since it recently has been reported that two genes encoding FTs with 81 % sequence identities did not share the same enzyme activity (Lasseur *et al.*, 2006).

During the last few years, the knowledge about FEHs has increased, but in *L. perenne* the information on FEHs is still incomplete. At the time, when the present PhD project was initiated, no genes encoding FEHs were isolated. To date, one full-length and one partial gene encoding 1-FEH-like enzyme activity have been isolated from *L. perenne*, but so far, no functional data are published. During this PhD study, six putative FEH genes have been isolated and performing an alignment, they all cluster with a group with 1-FEHs.

Two of the isolated putative FEH genes seem to be small splicing variants of FEHs, sharing 96 to 100 % sequence identity to *LpFEHa* in the 5' and 3' ends, but lacking 277 amino acids in the mid region compared to *LpFEHa*. Whether these genes encode for functional enzymes is so far unknown and it could be interesting to perform a functional characterisation of the isolated putative FEHs. An investigation of the transcription pattern in different plant tissues as well as under different climate conditions cold is very interesting.

Since the genomic clones of putative FEHs also have been isolated in this PhD project, it will be possible to design primers to screening a TILLING[1] population from *L. westerwoldicum* with mutations in genes encoding FEHs. If any mutants can be identified, the role of the FEHs and its participation in the fructan metabolism can be investigated further.

[1] Targeting Induced Local Lesions IN Genomes (TILLING) is a project initiated at DLF-TRIFOLIUM and has not be a part of the present PhD thesis.

6. Appendix

6.1 Poster presented at 5th International Fructan Symposium 2004, December 5 to 9, Havana, Cuba

Abstract

Analysis of fructan metabolism in *Lolium perenne.*

GITTE G. LARSEN, THOMAS DIDION AND KLAUS K. NIELSEN

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Increasing and stabilizing the amount of soluble carbohydrates at a high level throughout the season in forage crops has a great potential to increase the rates of milk and meat production together with benefits in terms of reduced loss of nitrogen to the environment.

In perennial ryegrass (*Lolium perenne*), like many others grasses, fructans are the major carbohydrate storage. In order to better understand fructan metabolism, genetic investigations are carried out. Not all the genes encoding for the enzymes catalysing fructan biosynthesis and/or -degradation in ryegrass are known and first step has been to isolate possible candidates genes. The isolated genes are currently being characterized in respect to sequence homology, protein domain structure and expression pattern. Subsequently, enzymatic properties will be determined by heterologous expression in *Pichia*.

In addition, expression studies will be performed in low and high sugar varieties (DLF-TRIFOLIUM) throughout the growth season combined with measurements of the water-soluble carbohydrate (WSC) content to identify the genes most active in fructan biosynthesis.

In a second approach heterologous fructosyltransferase genes have been transformed into *Lolium perenne* singly or in combinations with the aim of increasing fructan levels. The transgenic lines are analysed for presence of gene of interest and gene expression. Lines with transgene expression are currently being analysed for fructan and water-soluble carbohydrate (WSC) levels. The levels of WSC (glucose, fructose, sucrose and fructan) are assayed enzymatically using hexokinase, glucose-6-phosphate dehydrogenase and phosphoglucose isomerase after extraction and hydrolysis under acidic conditions.

ANALYSIS OF FRUCTAN METABOLISM IN *LOLIUM PERENNE*

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In perennial ryegrass (*Lolium perenne* L), like in many others grasses, fructans represent the major reserve of carbohydrate storage. Fructans are soluble polymers of fructose in linear or branched chains and stored primary in the stem base, but also in the leaves and roots [1]. The identification of fructosyltransferase, invertase and exohyrolase genes will provide a basis for expression studies and for analysis of transcriptional regulation of fructan biosynthesis genes. So far we have 1) isolated two 1-SST-like and three 6-FFT-like, two 6-SFT-like and two exohydrolase-like cDNAs from *L. perenne*, 2) transformed *Lolium perenne* with several heterologous fructosyltransferase genes singly and in combinations and 3) analyzed several DLF Trifolium elite varieties from field experiments in respect to sugar content and expression of fructosyltransferases and fructan exohydrolases.

1) Isolation of *Lolium perenne* genes involved in fructan metabolism

Screening of perennial ryegrass (*Lolium perenne* L) cDNA libraries and RACE based PCR techniques resulted the isolation of two 1-SST-like and three 6-FFT-like full-length (figure 1A), two 6-SFT-like partial (figure 1B) and two fructan exohydrolase-like (figure 1C) cDNA clones from *L. perenne.*

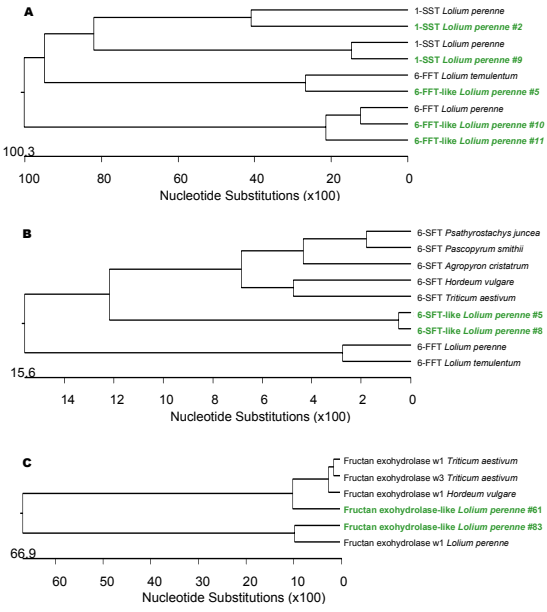

Figure 1. Unrooted phylogenetic three of isolated *Lolium perenne* genes.
A) 1-SST- like and 6-FFT-like full-length cDNA clones align to full-length *Lolium perenne* 1-SST and 6-FFT cDNAs.
B) 6-SFT-likes partial cDNA clones align to partial monocot cDNAs.
C) Fructan exohydrolase-like partial cDNA clones align to partial monocot cDNAs.

2) Transgenic approach

Heterologous fructosyltransferase genes from different mono- and dicots have been transformed into *L. perenne* singly or in combination with the aim to increase fructan levels. The transgenic lines have been analyzed for gene of interest (GOI) by PCR and for GOI expression by RT-PCR. All the lines with gene expression were cloned from one shoot and grown for 4-5 month prior to fructan measurements. The selected lines are currently being measured in respect to fructan content and composition.

3) Field experiments

Seven *Lolium perenne* varieties from DLF TRIFOLIUM were selected and sown out in 2002 and 2003. Through the season 2004 samples were harvested for measurement of Water Soluble Carbohydrates (WSC) content and for transcriptional expression studies of the genes involved in fructan biosynthesis. It is well known that fructan content decreases in grasses after defoliation [2,3] and recently it was reported that the first frost result in a decrease in fructan levels properly caused by an increased exohydrolase activity [4]. Samples from sowing year 2003 have been analyzed for sugar content (figure 2).

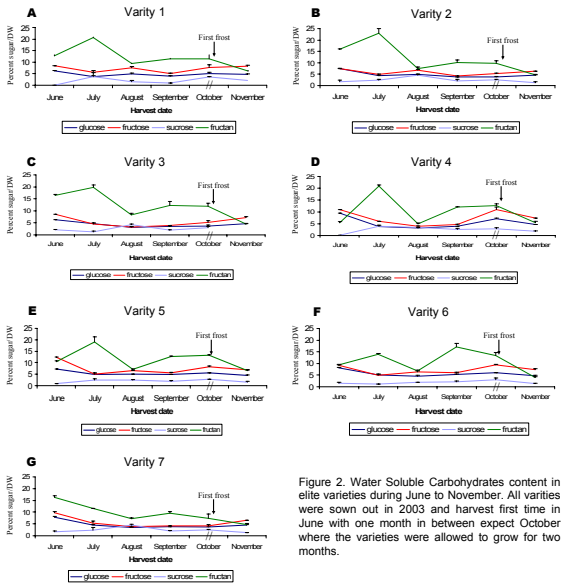

Figure 2. Water Soluble Carbohydrates content in elite varieties during June to November. All varities were sown out in 2003 and harvest first time in June with one month in between expect October where the varieties were allowed to grow for two months.

References

[1] Vijn and Smeekens (1999): Fructans: More than a Reserve Carbohydrate? Plant Physiol **120**, 351-359.
[2] Pavis *et al*., (2001): Structure of fructans in roots and leaf tissue of *Lolium perenne*. New Phytol. **150**: 83-95.
[3] Morvan-Bertrand *et al*., (2001): Roles of the fructans from leaf sheaths and from the elongating leaf base in the regrowth following defoliation of *Lolium perenne* L. Planta **213**: 109-120.
[4] Wilson *et al*., (2004): Chicory root yield and carbohydrate composition is influenced by cultivar selection, planting and harvest date. Crop Sci. **44**: 748-752.

6.2 Poster presented at 8th European Training Course on Carbohydrates 2004, 28 June to 1 July, Wageningen, the Netherlands

Abstract

Analysis of fructan metabolism in *Lolium perenne*.

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Increasing and stabilizing the amount of soluble carbohydrates at a high level throughout the season in forage crops has a great potential to increase the rates milk and meat production together with benefits in terms of reduced loss of nitrogen to the environment.

In perennial ryegrass (*Lolium perenne*), like many others grasses, fructans are the major carbohydrate storage. In order to better understand fructan metabolism, genetic investigations are carried out. Not all the genes encoding for the enzymes catalysing fructan biosynthesis and/or -degradation in ryegrass are known and a first step has been to isolate possible candidate genes. The isolated genes are currently characterized in respect to sequence homology, protein domain structure and expression pattern. Subsequently, enzymatic properties will be determined via heterologous expression in a yeast system. In addition, expression studies will be performed in low and high sugar varieties (DLF-TRIFOLIUM) throughout the growth season combined with measurements of the water-soluble carbohydrate (WSC) content to identify the genes most active in fructan biosynthesis.

In a second approach heterologous fructosyltransferase genes have been transformed into *Lolium perenne* singly or in combinations with the aim of increasing fructan levels. The transgenic lines are currently analysed for presence of gene of interest, gene expression and fructan and water-soluble carbohydrate (WSC) levels. The levels of WSC (glucose, fructose, sucrose and fructan) are assayed enzymatically using hexokinase, glucose-6-phosphate dehydrogenase and phosphoglucose isomerase after extraction and hydrolysis under acidic conditions.

ANALYSIS OF FRUCTAN METABOLISM IN *LOLIUM PERENNE*

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Introduction

Increasing and stabilizing the amount of soluble carbohydrates at a high level throughout the season in forage grasses has a great potential to enhance the rates of milk and meat production together with benefits in terms of reduced loss of nitrogen to the environment.

In perennial ryegrass (*Lolium perenne*), like many others grasses, fructans represent the major reserve of carbohydrate storage. Fructans are soluble polymers of fructose formed in the vacuoles (figure 1).

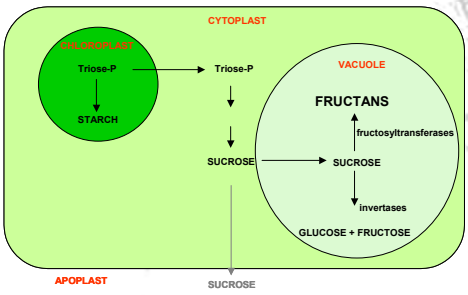

Figure 1: Fructan biosynthesis in the vacuole. Triose-P is converted to starch or transported from chloroplast to cytoplast where it is converted to sucrose. Sucrose is transported to the apoplast or vacuole where it is hydrolysed by invertases or used as substrate for fructan biosynthesis by fructosyltransferases. Modified from Vijn, I. And Smeekens, S. (1999): Fructans: More than a Reserve Carbohydrate? Plant Physiol. 120, 351-359.

Only a few genes encoding the enzymes catalysing fructan biosynthesis and/or -degradation in ryegrass have been isolated and the biosynthesis and -degradation pathway model is based on enzyme activities measured in grasses together with enzyme activities and isolated genes from other plants. Figure 2 illustrates a current model for fructan biosynthesis in plants.

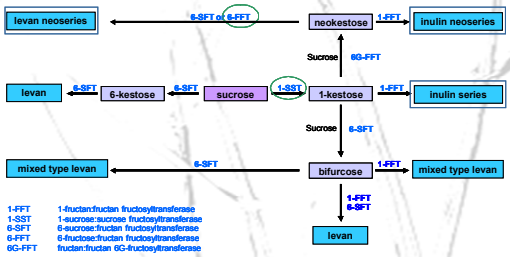

Figure 2: Model for fructan biosynthesis in plants. Sucrose (dark purple) is the key substrate and basis for the biosynthesis of the different fructosyltransferases. Light purple boxes symbolise substrates formed and blue boxes different fructan types. Dark blue boxes illustrate fructan types found in *L. perenne* and green circles represent fructosyltransferases isolated from *L. perenne*. Modified from Pavis *et al.* (2001): Fructans and fructan-metabolizing enzymes in leaves of *Lolium perenne*. New Phytol. 150, 97-109.

Beside hydrolysis of the sucrose unit by invertases in the vacuole (figure 1), fructans are subject to degradation by exohydrolases. So far, no full-length fructan exohydrolases have been isolated from *L. perenne* but the presence of vacuolar exohydrolases in other plants has been reported.

In addition from a role in photosynthetic partitioning, fructan accumulation has been associated with plant response to environmental stresses such as cold or drought.

Results

Genes involved in fructan metabolism

Isolation of fructosyltransferase, invertase and exohyrolase genes provides a basis for expression studies in order to identify the rate limiting enzymes and the transcriptional regulation of fructan biosynthesis.

Tabel 1: Percent homology of isolated genes

Gene	Partial or full-length	Percent homology in BlastX
1-SST	Full-length	67% to AAO86693.1 sucrose:sucrose 1-fructosyltransferase [Lolium perenne]
	Partial	93% to AAM13671.1 putative sucrose:sucrose 1-fructosyltransferase [Lolium perenne]
6-FFT	Full-length	92% to AAM14603.1 fructan 6-fructosyltransferase [Lolium perenne]
	Full-length	64% to AAM14603.1 fructan 6-fructosyltransferase [Lolium perenne]
	Full-length	60% to AAM14603.1 fructan 6-fructosyltransferase [Lolium perenne]
6-SFT	Partial	95% to CAA58235.1 sucrose:fructan 6-fructosyltransferase [Hordeum vulgare subsp.vulgare]
	Partial	73% to AAG24788.1 sucrose:fructan 6-fructosyltransferase [Pascopyrum smithii]
Exohydrolase	Partial	64% to CAD92365.1 fructan 1-exohydrolase w3 precursor [Triticum aestivum]
	Partial	73% to CAD48199.1 fructan 1-exohydrolase [Triticum aestivum]
Acid invertase	Partial	87% to CAD58681.1 putative soluble acid invertase [Lolium temulentum]

Table 1 summarizes the genes isolated so far and shows sequence homology to published sequences. Sequence information will be used to design gene specific primers for RT-PCR studies to investigate the expression pattern in *L. perenne*.

Expression of heterologous fructosyltransferases increase fructan levels in *Lolium perenne*

In a second approach heterologous fructosyltransferase genes have been transformed into *L. perenne* singly or in combinations in order to increase fructan levels (figure 3).

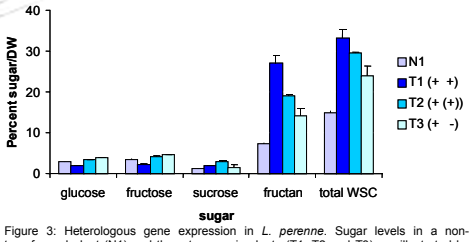

Figure 3: Heterologous gene expression in *L. perenne*. Sugar levels in a non-transformed plant (N1) and three transgenic plants (T1, T2 and T3) are illustrated by percent sugar/dry weight. T1 expresses two heterologous genes encoding fructosyltransferases at a high level (+ +). T2 expresses one heterologous fructosyltransferase at a high level and one at a low level (+ (+)). T3 expresses only one heterologous fructosyltransferase (+ -).

Figure 3 illustrates a significant increase in fructan levels. The fructan levels correlate to the transgene expression levels, showing highest fructan content when two genes are highly expressed as determined by RT-PCR.

Summary and perspective

Four full-length fructosyltransferase-like cDNA's have been isolated and will be characterized with respect to expression pattern and enzyme activity.

Investigation of *Lolium perenne* plants transformed with four fructosyl-transferases will uncover if a further increase in fructan level is possible.

Finally, gene expression of fructosyltransferases, invertases and exohydrolases will be investigated during the growth season 2004 in eights DLF-TRIFOLIUM elite varieties and correlated to the content of fructans.

6.3 Alignment of LpFTa

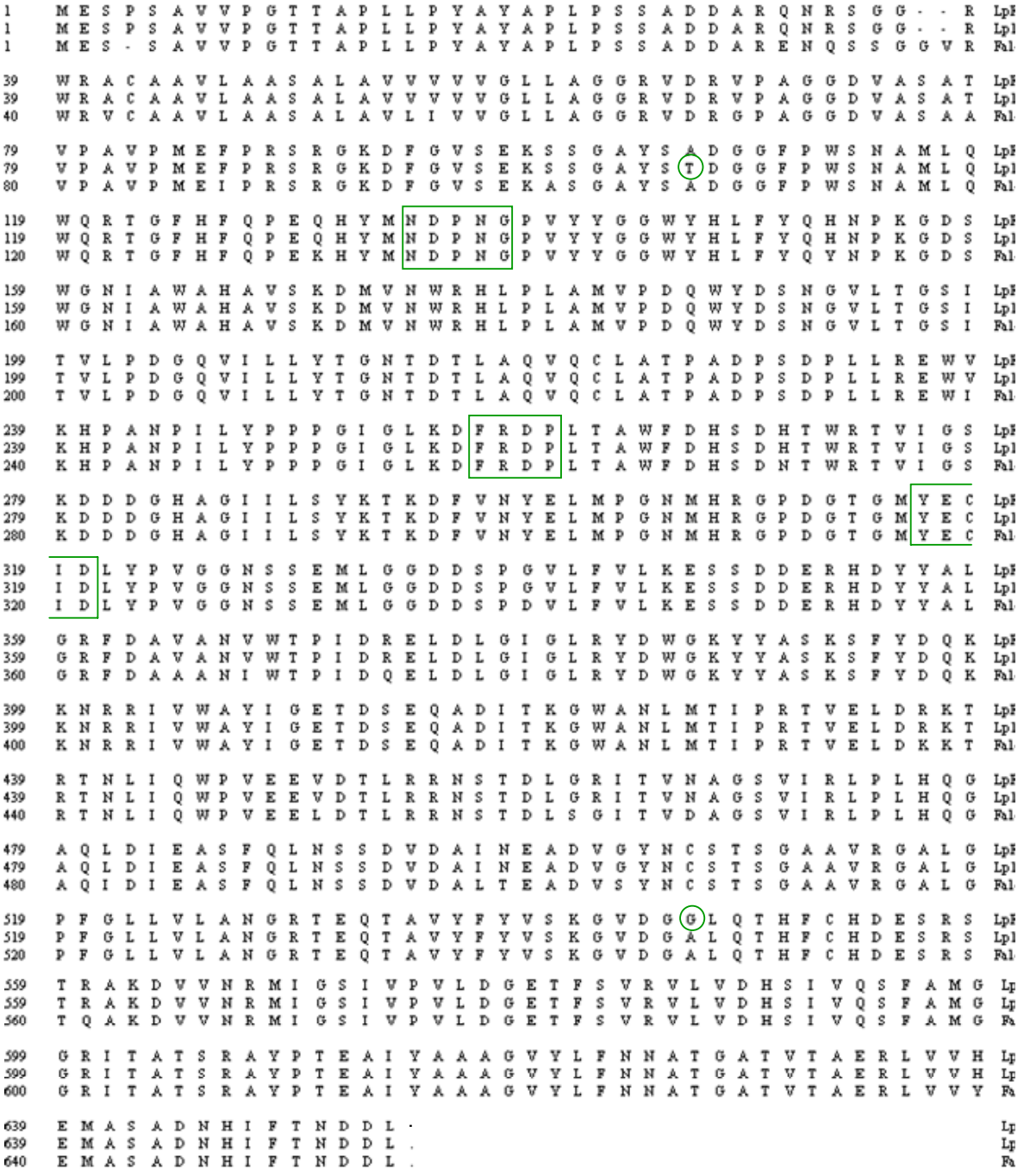

Figure 6.1 Alignment of the deduced amino acids sequences of *LpFTa* to *Lp1-SST* and *Fa1-SST*. Their respective accession numbers are: *LpFTa*, DQ408726; *Lp1-SST*, AY245431 and *Fa1-SST*, AJ297369. The amino acids which differ from the majority are marked with a green circles. The NDPNG, FRDP and WECPD motifs are marked with a green square.

6.4 Alignment of LpFTb

Figure 6.2 Alignment of the deduced amino acids sequences of *LpFTb* to *Lp6-FT*, *Lp1-FFT* and *Lt6-FT*. Their respective accession numbers are: *LpFTb*, DQ408727; *Lp6-FT*, AF494041, *Lp1-FFT AB186920* and *Ft6-FT*, AJ532550. The amino acids which differ from the majority are marked with a green circles. The NDPNG, FRDP and WECPD motifs are marked with a green square.

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